REMARKS

Claim Amendments

Claims 1-80 have been canceled. New Claims 81-122 have been added.

Support for new Claims 81 and 85 can be found in the specification, for example, at page 2, lines 6 and 7; page 8, lines 3-4; and page 10, lines 12-14.

New Claims 82-84 find support in the specification, for example, at page 13, lines 9-11 and 26-28.

Support for new Claims 86 and 87 can be found in the specification, for example, at page 10, lines 13 and 14.

New Claims 88-95 and 97 correspond to canceled Claims 9-17, except they are dependent from new Claim 81.

Support for new Claims 96 and 98 can be found in the specification, at page 11, lines 25-29.

Support for new Claim 99 can be found in the specification, at page 12, lines 3-4.

New Claims 100-104 correspond to canceled Claims 27-31, except they are dependent from new Claim 99.

New Claims 105-107 and 109-110 correspond to canceled Claims 23-25 and Claims 2-3 respectively, except that they are dependent from new Claim 81.

Support for new Claim 108 can be found in the specification, for example, at page 5, lines 8-14.

Page 13, lines 9-16 describes that a PHA solution can be applied to a substrate surface and some or all of the solvent can then be removed to form a layer of a PHA adhesive composition on the substrate surface, providing support for Claims 111-114.

Support for new Claims 115-117 can be found in the specification, for example, at page 13, lines 26-28 and page 14, lines 16-18.

Support for new Claim 118 can be found, for example, at page 10, lines 12-14.

Support for new Claims 119-122 can be found in the specification, for example, at page 3, lines 13-19; page 8, lines 3-4; and page 10, lines 12-14.

No new matter has been added.

Rejection of Claims 1-16, 20-36, 40-46, 48-55, 57-71 and 74-80 under 35 U.S.C. §102(b)

Claims 1-16, 20-36, 40-46, 48-55, 57-71 and 74-80 were rejected under 35 U.S.C. §102(b) as being anticipated by Hammond (U.S. Patent No. 5,646,217).

Applicant's Claimed Invention

Claim 81 is directed to an article comprising a specific PHA adhesive composition supported by a surface of the article; and Claim 111 is directed to a method of applying the adhesive composition to the surface of a substrate.

Claim 84 is directed to an article comprising two substrate surfaces bonded together with the PHA adhesive composition; and Claim 115 is directed to a method of binding the two substrate surfaces together with the PHA adhesive composition. Claim 119 is directed to a method of pressing a PHA between at least two surfaces for form a pressed PHA

Applicant has discovered that certain poly 3-hydroxybutyrate-co-4-hydroxybutyrate copolymers have unexpected adhesive properties, and, therefore, are suitable for use in an adhesive composition. For example, Examples 13-24 of the present application describe unexpected adhesive properties of poly 3-hydroxybutyrate-co-4-hydroxybutyrate copolymers having glass transition temperature of -10 °C, -14 °C and -26 °C. Specifically, in these examples poly 3-hydroxybutyrate-co-4-hydroxybutyrate copolymers were used to laminate two films together. It is noted that Examples 3-9 describe that other polyhydroxyalkanoate (PHA) polymers have poor adhesive qualities.

The glass transition temperature of a poly 3-hydroxybutyrate-co-4-hydroxybutyrate copolymer correlates with the amount of 4-hydroxybutyrate comonomer present in the polymer (see Sudesh, K. *et al.* (Exhibit A) at page 1529, Fig. 10). As shown in Fig. 10 of Sudesh *et al.*, the glass transition temperature has a linear correlation with the amount of 4-hydroxybutyrate comonomer present. Based on this linear correlation, a copolymer with a glass transition temperature in the range of -30 °C to -5 °C corresponds to a copolymer having about 15-70% of 4-hydroxybutyrate comonomer.

The Teachings of Hammond

Hammond teaches a polymer composition comprising "a first component which is a microbiologically produced polyhydroxyalkanoate (PHA) and a second component which is a different microbiologically produced PHA or is a synthetic polyester, and at least one transesterification catalyst." (see Column 1, lines 19-27) Hammond also teaches that suitable PHAs have units of formula I:

$$-O-C_mH_n-CO-$$

and "particular polyesters contain a preponderance of m=3 units, especially with 70-95 mol% of such units, the balance being units in which m=4" (Column 1, lines 40-47).

In the Office Action of April 28, 2008, the Examiner stated that m=4 represents 4-hydroxybutyrate monomer (see page 2, line 6 of paragraph 3 in the Office Action). Applicant respectfully submits that the Examiner's interpretation is incorrect and m=4 represents valerate monomer, not 4-hydroxybutyrate monomer. Valeric acid by definition has 5 carbon atoms, i.e. C_4H_9COOH and m=4. (see Hawley's Condensed Chemical Dictionary; 12th edition, Van Nostrand Reinhold, New York, NY, Exhibit B). In addition, column 1, lines 53-55 of Hammond states:

> ...and PHA consisting m=3 and 4 units is polyhydroxybutyrate-co-valerate (PHBV). (emphasis added)

Furthermore, all of the working examples in Hammond describe PHA blends containing a polyhydroxybutyrate-co-valerate copolymer. Therefore, Hammond does not specifically teach poly 3-hydroxybutyrate-co-4-hydroxybutyrate copolymer.

Hammond does not teach, suggest or motivate the use of poly 3-hydroxybutyrate-co-4-hydroxybutyrate copolymer, let alone that poly 3-hydroxybutyrate-co-4-hydroxybutyrate copolymer with the recited glass transition temperatures would have adhesive properties. Moreover, there would be no reason to apply these copolymers to a substrate, as required by the instant claims, if one were unaware of these adhesive properties. Therefore, the claimed subject matter is novel and non-obvious in view of Hammond.

Claims 84, 115 and 119 are non-obvious for yet other reasons. These claims are directed to an article comprising two substrate surfaces bonded together with the adhesive poly 3-hydroxybutyrate-co-4-hydroxybutyrate copolymer composition described above and to methods of bonding two substrate surfaces together with this adhesive composition. One of ordinary skill

in the art would not know that the adhesive poly 3-hydroxybutyrate-co-4-hydroxybutyrate copolymer composition could be used for this purpose without a teaching that it has adhesive properties. Because Hammond does not supply this teaching, new Claims 84, 115 and 119 and claims depending therefrom are novel and non-obvious in view of Hammond.

Rejection of Claims 37-39, 47, 56, 72 and 73 under 35 U.S.C. §103(a)

Claims 37-39, 47, 56, 72 and 73 were rejected under 35 U.S.C. §103(a) as being unpatentable over Hammond.

Claims 37-39, 47, 56, 72 and 73 have been canceled, thereby obviating the rejection.

Rejection of Claims 17-19 under 35 U.S.C. §103(a)

Claims 17-19 were rejected under 35 U.S.C. §103(a) as being unpatentable over Hammond in view of Procter & Gamble (WO 97/04026).

Claims 17-19 have been canceled, thereby obviating the rejection.

Double Patenting

Claims 1-80 were rejected or provisionally rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over Claims 1-67 of U.S. Patent No. 6,780,911, Claims 1-54 of U.S. Patent No. 7,094,840, Claims 1-82 of co-pending Application No. 10/783,995, Claims 1-55 of co-pending Application No. 09/999,769 and Claims 43-90 of co-pending Application No. 11/479,360.

U.S. Patent No. 6,780,911 (herein after "the '911 patent")

Claims 1-80 were rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 1-67 of U.S. Patent No. 6,780,911 in view of Hammond.

Claims 1-67 of the '911 patent are directed to a composition or a molding composition comprising at least one thermally decomposable polyhydroxyalkanoate having a molecular weight in a specific range and method of using and method of making thereof. Column 2, lines 58-62 clarifies the meaning of "molding composition", stating that "[t]he use of

polyhydroxyalkanoates as a binder in molding compositions provides improved binder removal in the finished molded product, and offers a wide range of physical properties suitable for use in a variety of processing conditions". Column 12, lines 8-20 further describes that the polyhydroxyalkanoate binder can be preferably removed by thermal decomposition. Therefore, the polyhydroxyalkanoate to be used in the molding composition needs to is necessarily to be thermally decomposable. As such, the use of polyhydroxyalkanoates in molding compositions does not teach or suggest adhesive properties. Therefore, there is no teaching, suggestion or motivation in the claims of the '911 patent for one of ordinary skill in the art to apply poly 3-hydroxybutyrate-co-4-hydroxybutyrate copolymers having a glass transition temperature from about -30 °C to about -5 °C (i.e. having about 15-70% of 4-hydroxybutyrate comonomer) to a surface (as in Claims 81 and 11). More particularly, it provides no reason to use the poly 3-hydroxybutyrate-co-4-hydroxybutyrate copolymers to bond two substrate surfaces together, as required in Claims 84, 115 and 119.

Hammond does not cure the deficiencies of the claims in the '911 patent for a number of reasons. Hammond does not teach poly 3-hydroxybutyrate-co-4-hydroxybutyrate copolymer, let alone poly 3-hydroxybutyrate-co-4-hydroxybutyrate copolymer having the specific amount of 4-hydroxybutyrate comonomer required by the instant claims. Moreover, it does not teach that these poly 3-hydroxybutyrate-co-4-hydroxybutyrate copolymers have adhesive properties. Thus, Applicant's claimed invention, as set forth in new Claims 81-122, are non-obvious over the claims of the '911 patent in view of Hammond and the non-statutory obviousness-type double patenting rejection should be withdrawn.

U.S. Patent No. 7,094,840 (hereinafter "the '840 patent")

Claims 1-54 of the '840 patent are directed to hot melt adhesive compositions comprising at least one polyhydroxyalkanoate having a molecular weight between about 500 to 50,000, and method of use thereof. However, there is no teaching, suggestion or motivation in any of the claims in the '840 patent for one of ordinary skill in the art to select and make the specific poly 3-hydroxybutyrate-co-4-hydroxybutyrate having a glass transition temperature from about -30 °C to about -5 °C (i.e. about 15-70% 4-hydroxybutyrate comonomer), as required in the instant claims. As noted in the section of this Paper entitled "Applicant's Claimed Invention",

Examples 15, 18 and 19 of the instant application exemplify the good adhesive properties of poly 3-hydroxybutyrate-co-4-hydroxybutyrate copolymers having a glass transition temperature from about -30 °C to about -5 °C (about 15-70% of 4-hydroxybutyrate). No hot melt process is required. In contrast, Examples 3-9 provide examples where other polyhydroxyalkanoate polymers were used and shown to have poor adhesive qualities. Therefore, the instant claims select those poly 3-hydroxybutyrate-co-4-hydroxybutyrate copolymers that have adhesive properties. Without a knowledge of which poly 3-hydroxybutyrate-co-4-hydroxybutyrate copolymers have adhesive properties, one of ordinary skill in the art would not apply these poly 3-hydroxybutyrate-co-4-hydroxybutyrate copolymer to a surface, as required by Claims 81 and 111. Moreover, without this knowledge, one of ordinary skill in the art would not use these poly 3-hydroxybutyrate-co-4-hydroxybutyrate copolymers to bond two surfaces together, as required by Claims 84, 115 and 119. Therefore, Applicant's claimed invention is not an obvious variation of Claims 1-54 of the '840 patent.

Furthermore, it is submitted that the entire teachings of the '840 patent do not add to the teachings of its claims. Therefore, Applicant's claimed invention is also not obvious in view of the entire teachings in the '840 patent.

U.S. Application No. 10/783,995 (hereinafter "the '995 application")

Claims 1-80 were provisionally rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over Claims 1-82 of copending Application No. 10/783,995.

Claims 1-82 of the copending '995 application have been canceled and new Claims 86-113 have been added (see Amendment filed by Applicant's agent on July 9, 2008 in response to Office Action dated January 10, 2008). New Claims 86-113 of the '995 application are directed to a blend comprising three PHAs and an article composition comprising the blend. Therefore, new Claims 81-121 of the instant application are not obvious variations of the pending claims in the '995 application.

CONCLUSION

In view of the above amendments and remarks, it is believed that all claims are in condition for allowance, and it is respectfully requested that the application be passed to issue. If the Examiner feels that a telephone conference would expedite prosecution of this case, the Examiner is invited to call the undersigned.

Respectfully submitted,

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PROGRESS IN POLYMER SCIENCE

Synthesis, structure and properties of polyhydroxyalkanoates: biological polyesters

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Abstract

High molecular weight polyhydroxyalkanoates (PHA) are synthesized and stored in the cell cytoplasm as water-insoluble inclusions by various microorganisms. This intriguing biological polyester initially attracted the attention of microbiologists and managed to keep many polymer scientists occupied over the second half of the last century. Concerted multidisciplinary scientific approaches have been directed to the elucidation of various aspects of PHA. Of significant interest are the findings that PHA can consist of various hydroxyalkanoate monomers, and the cloning of its biosynthesis genes. This has resulted in the production of PHA with various physical properties by genetically engineered microorganisms. In fact, it is now possible that large-scale production of PHA by transgenic plants can be achieved in the near future. The physical properties of PHA homopolymers as well as co- and heteropolymers have been the subject of study in various laboratories all over the world. By controlling the monomer composition of PHA, polymer scientists have shown that the polymer's physical properties can be regulated to a great extent. Furthermore, it is also clear that the rate of degradation of PHA in various environments can be controlled by judiciously altering its monomer compositions. This review attempts to bring together the biochemical and physicochemical aspects of PHA along with new perspectives on its potential therapeutic applications.

Keywords: Polyhydroxyalkanoates; Polyhydroxybutyrate; 4-Hydroxybutyrate (GHB); Polyesters; PHA synthase; Biodegradable; Biocompatible; Freeze-fracture; Plastic deformations; Polymer blends; Solid-state structure; Single crystals; Enzymatic degradation

Contents

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1. Introduction: history of polyhydroxyalkanoates (PHA)

The presence of sudanophilic, lipid-like inclusions [1] which were soluble in chloroform [2] was initially observed in *Azotobacter chroococcum* early last century. The chemical composition of similar inclusions in *Bacillus megaterium* was later identified as poly(3-hydroxybutyric acid) (P[3HB]) by Lemoigne [3,4]. By the end of the 1950s, enough evidence was already accumulated from studies on the genus *Bacillus* to suggest that P(3HB) functions as an intracellular reserve for carbon and energy in these bacteria [5–7]. In fact, it was also demonstrated that the occurrence of this reserve polymer is a widespread phenomenon in Gram-negative bacteria [8]. In a review on the role and regulation of energy reserve polymers in microorganisms published in 1973 [9], P(3HB) received its first extensive coverage as a bacterial storage material analogous to starch and glycogen. To this end however, the 3-hydroxybutyrate (3HB) unit was thought to be the only hydroxyalkanoate (HA) constituent that forms the building block for this microbial reserve polymer.

In 1974, Wallen and Rohwedder reported the identification of hydroxyalkanoates (HA) other than 3HB [10]. Among the HA units that were noted in chloroform extracts of activated sewage sludge, 3-hydroxyvalerate (3HV) and 3-hydroxyhexanoate (3HHx) were the major and the minor constituents, respectively. About a decade later, following the identification of the heteropolymers, the analysis of marine sediments by capillary gas chromatography revealed the presence of 3HB and 3HV as the predominant components among 11 other HA units [11]. In the same report it was also shown that batch-grown *Bacillus megaterium* cells accumulated a polymer which consisted of 95% 3HB, 3% 3-hydroxyheptanoate (3HHp), 2% of an 8-carbon HA and trace amounts of three other HA compounds.

At around the same time (1983), an interesting finding was made by Witholt and coworkers when they cultivated *Pseudomonas oleovorans* on *n*-octane [12]. Elemental analysis of the storage polymer thus accumulated by this bacterium showed that it consisted of principally 3-hydroxyoctanoate (3HO) units [12] and small amounts of 3HHx units [13].

The identification of HA units other than 3HB in microbial PHA proved to have major impact on the research and commercial interest for this bacterial reserve polymer. While the homopolymer of P(3HB) is a brittle material with limited applications, the incorporation of a second monomer unit into P(3HB) can significantly enhance its useful properties. This finding is therefore highlighted as a landmark which signifies the beginning of the second developmental stage of research on PHA (Fig. 1). It was in this stage that the first industrial production of a copolymer consisting of 3HB and 3HV took place [14], despite the fact that the potential of P(3HB) as a biodegradable thermoplastic was realized much earlier and patents were originally filed in 1962 [15]. In this stage (1980s), the research trend was to identify and characterize all the various potential HA units that could possibly be a constituent of this bacterial polyester. This ultimately resulted in the discovery of numerous HA constituents [16] including 4- [17] and 5HA [18] by the end of the 1980s. By this time it was already clear that these storage polymers are synthesized not only in Gram-negative bacteria but also in a wide range of Gram-positive bacteria, aerobic (cyanobacteria) and anaerobic (non-sulfur and sulfur purple bacteria) photosynthetic bacteria, as

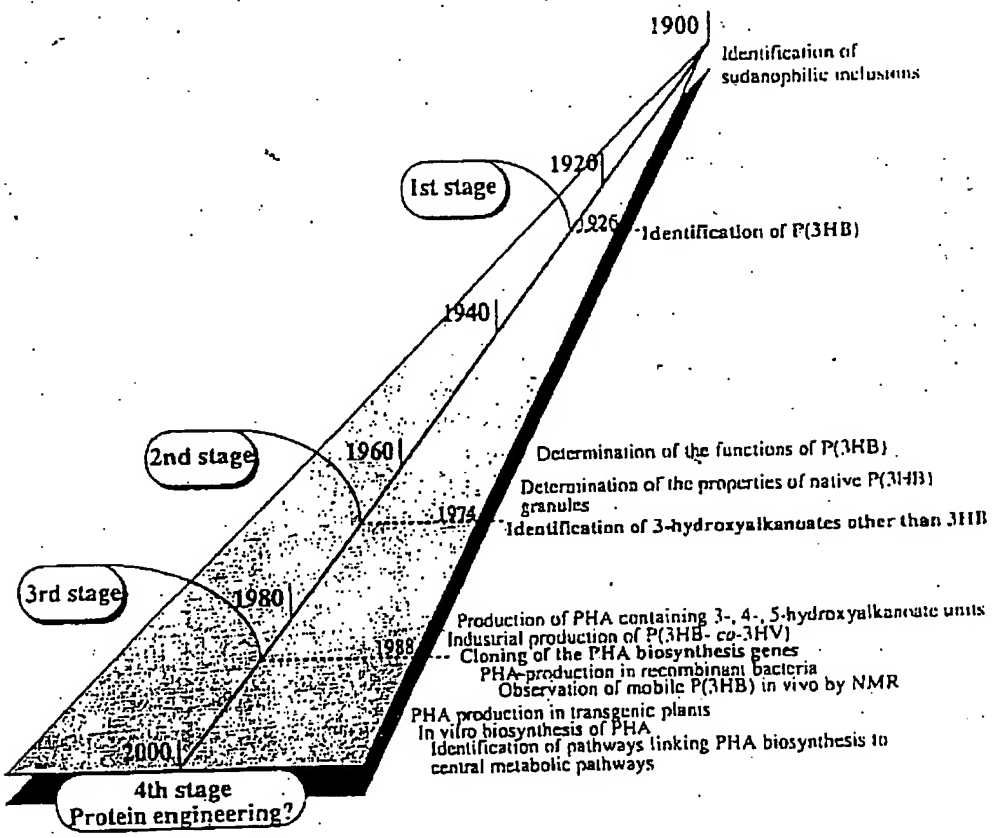


Fig. 1. The development of PHA science and technology through the twentieth century.

well as in some archaebacteria [19,20]. To date however, an astounding number of approximately 125 different HA are known to occur [21], and therefore a more general name comprising all these constituents, i.e polyhydroxyalkanoates (PHA), has been used to designate this family of bacterial reserve polymers.

The third stage of development in research concerning bacterial PHA involved the cloning and characterization of genes involved in the biosynthesis of reserve polyesters. The onset of the molecular biology revolution during the late 1970s provided new tools for biological research, which were successfully used to decipher genetic information and to understand further the principles behind PHA biosynthesis at the genetic level. By the end of the 1980s, the genes coding for enzymes involved in PHA biosynthesis were already cloned from *Ralstonia eutropha* (formerly known as *Alcaligenes eutrophus*) and the genes were also shown to be functionally active in *Escherichia coli* [22–25].

Detailed studies on *R. eutropha* had revealed that only three enzymes are involved in the biosynthesis of P(3HB) from acetyl-CoA, and that the regulation of P(3HB) synthesis in this bacterium is achieved at the enzyme level. The enzyme which carries out the polymerization reaction was identified as the key enzyme and it was designated as PHA synthase. To date, about 38 PHA synthase structural genes from more than 32 different bacteria have been cloned. These studies show that the enzymes can be categorized into three major groups based on their primary structures and substrate specificities [21]. The recent knowledge that some of the active PHA synthases consist of two subunits has also been taken into consideration for the classification process. Highly conserved amino acids have been identified based on alignment analysis of the primary structures of these genes and also by site-specific mutagenesis studies [26]. The successful cloning of PHA biosynthetic genes had also enabled the generation of transgenic plants that will most likely be the potential producers of PHA in the future [27].

At present, the topic of interest involves the determination of tertiary and quaternary structures of PHA synthases, which would enable us to understand the catalytic mechanisms, and the substrate specificities of this group of enzymes, and probably also the factors that determine the molecular weight of PHA produced.

What was identified in the beginning of the twentieth century as a sudanophilic bacterial inclusion, is now apparently going into its fourth stage of development, i.e. protein engineering. This stage would determine whether it would be possible for mankind to manipulate the PHA synthase and other enzymes involved in the biosynthesis of PHA to tailor-make useful environmentally friendly polymers in an efficient manner.

2. Part I: biological aspects of PHA

2.1. The nature of PHA inclusions

PHA are a family of optically active biological polyesters which contain (*R*)-3HA monomer units [19]. The 3-hydroxyalkanoic acids are all in the *R* configuration due to the stereospecificity of the polymerizing enzyme, PHA synthase. Only in one rare case were a small portion of the *S* monomers detected [28]. The most well-known member of PHA is P(3HB), containing repeat units of (*R*)-3HB. The monomers are polymerized into high molecular weight polymers in the range of 200,000 to 3,000,000 Da, depending on the microorganism and growth conditions [29]. Recently, P(3HB) with a

number-average molecular weight (M_n) of up to 20 MDa was produced by using a recombinant strain of *E. coli* [30].

PHA exist as discrete inclusions that are typically 0.2–0.5 μm in diameter localized in the cell cytoplasm and may be visualized quite clearly with a phase contrast light microscope due to their high refractivity [9]. When thin sections of PHA-containing bacteria are observed by transmission electron microscopy, the PHA inclusions appear as electron-dense bodies as can be seen in Fig. 2. Native PHA inclusions can be stained with Sudan black B [31] indicating that they are of a lipid nature [5,32]. It was also noted that purified polymer and inclusions that were isolated under rigorous conditions do not take up this dye, presumably because their surrounding membranes have been removed during purification [9]. Besides Sudan black B, PHA is more specifically stained by the oxazine dye Nile Blue A, exhibiting a strong orange fluorescence at an excitation wavelength of 460 nm [33]. Recently, it was demonstrated that Nile Blue A and its fluorescent oxazone form, Nile Red can be used to detect PHA directly in growing bacterial colonies [34]. While staining methods can be used to identify the presence of PHA, chemical analysis is often required to determine their monomeric compositions. Gas chromatography (GC) and nuclear magnetic resonance (NMR) spectroscopy analysis are widely used for this purpose.

2.1.1. *The physical state of in vivo PHA inclusions*

Until the mid 1980s, the prevailing believe was that PHA in vivo is a crystalline solid [35,36]. Using solution-state NMR techniques, the group of Sanders [37,38] was able to demonstrate for the first time that the bulk of P(3HB) in vivo is not crystalline and indeed is a mobile amorphous polymer. This explains why mild treatments such as centrifugation lead to the rapid and irreversible loss of its

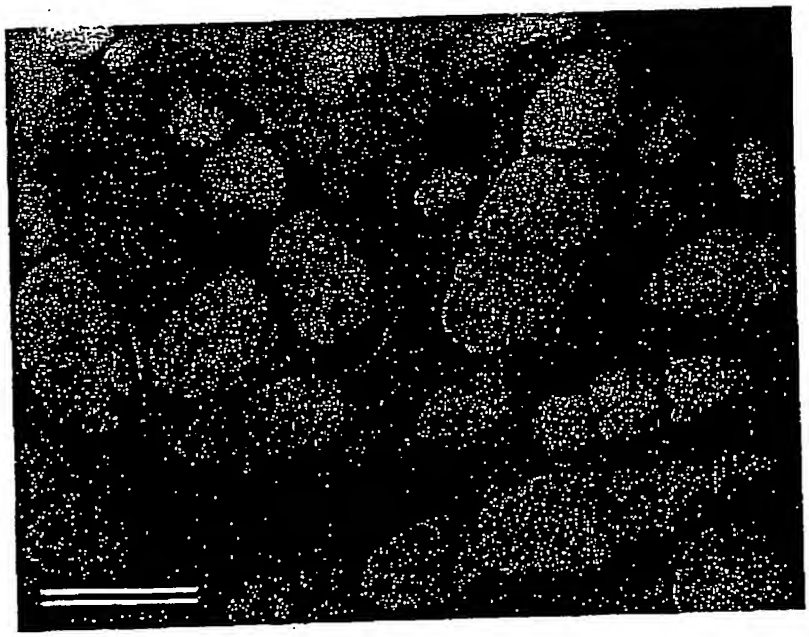


Fig. 2. Transmission electron micrograph of thin sections of recombinant *R. eutropha* PHB⁻4 cells containing large amounts (90% of the dry cell weight) of P(3HB-*co*-5 mol% 3HHx). Bar represents 0.5 μm.

degradability by the intracellular depolymerizing enzyme. Many of the treatments which lead to biological inactivation of the P(3HB) inclusion also lead to loss of the high-resolution NMR spectrum and the simultaneous appearance of the characteristic X-ray powder diffraction pattern of crystalline P(3HB) [39,40]. The conclusion that PHA in vivo is present as a mobile liquid resolved some of the earlier misconceptions, but at the same time raised new questions about the possible existence of plasticizers or nucleation inhibitors within the inclusion to prevent crystallization in vivo. It was also of interest to determine what triggers crystallization when the inclusions are isolated.

One plausible explanation for this lies in the physics of crystallization [41] and the support for this hypothesis was obtained by showing that it is possible to prepare stable amorphous P(3HB) inclusions in vitro [42]. Based on this observation, the PHA in vivo was thought to be under kinetic, and not thermodynamic control. Therefore, no specific plasticizers would be required to prevent crytallization in vivo. However, when PHA inclusions are subjected to physical treatments such as centrifugation, they readily coalesce into larger masses and this can lead to the apparent acceleration of crystallization [40]. Furthermore, any damage to the surface coating of the inclusion will allow heterogeneous nucleation, i.e. crystallization induced by external molecules other than PHA, further accelerating crystallization [41].

An intriguing phenomenon (Fig. 3) can be observed when cells containing PHAs are subjected to freeze-fracture analysis as noted initially in the freeze-fracture preparations of *Bacillus cereus* [43], *Rhodopseudomonas viridis* [44] and *Nitrobacter winogradskyi* [45]. When the freeze-fracture process was carried out at temperatures between −173 and −100°C, a typical needle-like deformation of P(3HB) inclusions was observed [46,47]. However, if fracturing takes place in the presence of liquid nitrogen (−196°C), the P(3HB) inclusions were found to deform into a mushroom-like structure, and

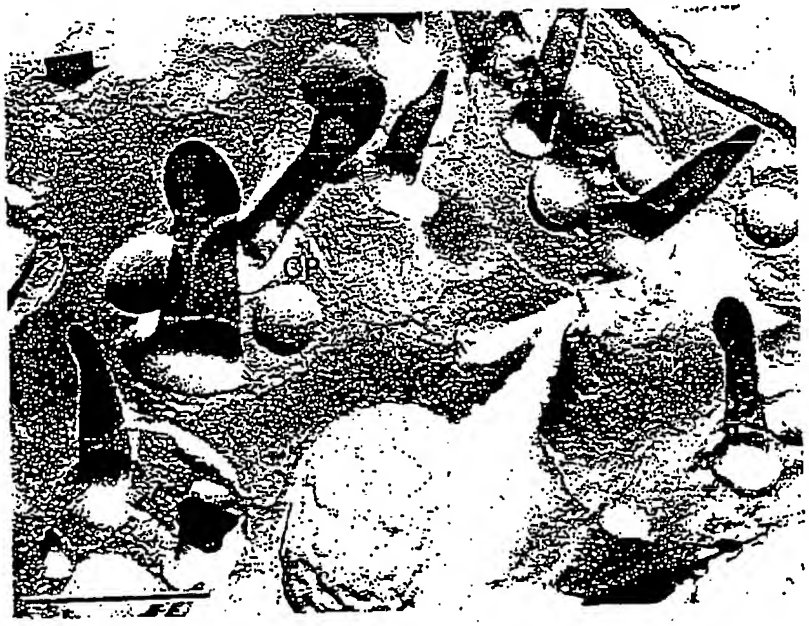


Fig. 3. Freeze-fracture electron micrograph of recombinant *R. eutropha* PHB⁻4 cells containing about 50% of the dry cell weight P(3HB) homopolymer. Fracturing was carried out at −160°C. CP, cytoplasm; arrow indicates direction of platinum-carbon shadowing; bar represents 0.5 μm.

this becomes the predominant form when fracturing was carried out in liquid helium (−269°C) [47]. In the field of polymer science, the phenomena of elastic and plastic deformation are well known [48]. The reversible extension of a polymer is elastic deformation (e.g. rubber), and it is probable that there is an elastic component to deformation during freeze-fracture. On the other hand, deformation of materials that have little elasticity results in either rupture or plastic flow, which leads to plastic deformation. When an elastic or plastic polymeric material is cooled below its glass transition temperature (T_g), it may no longer be able to dissipate energy by dimensional changes, and will tend to exhibit brittle fracture. A polymer below its T_g is then in its glassy state where it loses its ductility.

Some typical T_gs of PHAs and chemosynthesized polymers have been summarized in Table 1. The plastic deformation of polymers listed in Table 1 is interesting because it occurs at temperatures of −100°C or lower, which is far below the T_g of most of the polymers. The study of freeze-fracture on chemically and morphologically defined latex spheres gave clear indications of plastic deformations [44,49]. Further analysis using polyacrylate-latex spheres (0.2 μm) that were freeze-fractured at various temperatures (−100, −196 and −269°C) showed that there was less deformation when these systems were fractured at −196°C and almost no determinable deformation at −269°C. This showed that the freeze-fracture deformation of at least some polymers is dependent on the temperature at which the fracturing process is carried out [47]. Recent freeze-fracture studies on the various PHA inclusions in *Comamonas acidovorans* clearly shows that, besides the freeze-fracture temperature, the monomer composition of PHA can also greatly influence the extent of deformation [50].

It is now known that the in vivo PHA inclusions are largely amorphous [51], but what prevents crsytallization is still ambiguous. Water is a minor component of PHA inclusions and therefore it was suggested that water could act as a plasticizer [38,52]. In a model attempting to explain the polymerizing mechanism of PHA synthase, water was also proposed to be the chain transfer agent [53]. About 5–10% of water was estimated to be present in the nascent P(3HB) inclusions which upon removal allows for the polymer chains to rearrange into lamellar crystals [54]. Based on this finding, it was suggested that water molecules could form hydrogen bonds with the carbonyl groups of the polyester backbone to form "pseudo cross-links" between adjacent polymer chains. This type of molecular arrangement may also explain the mobile amorphous nature of PHA inclusions as well as the plastic deformation phenomenon [46,50,55] observed in freeze-fracture experiments [54].

Table 1
Thermal properties of some biosynthesized and chemosynthesized polymers (3HB, 3-hydroxybutyrate; 3HV, 3-hydroxyvalerate; 4HB, 4-hydroxybutyrate; PHA_{MCL}, PHA with mainly medium-chain-length monomers ranging from C_6–C_{12})

	Melting temperature, T_m (°C)	Glass-transition temperature, T_g (°C)	Reference
P(3HB)	180	4	[16]
P(3HB-*co*-71% 3HV)	83	−13	[16]
P(4HB)	53	−48	[130]
PHA_{MCL}	45–54	−25 to −40	[331]
Polyacrylate	–	−106	[332]
Polypropylene	176	−10	[16]
Polystyrene	240	100	[332]

2.1.2. Proteins associated with PHA inclusions

The surface of in vivo PHA inclusions is a dynamic boundary region where the proteins involved in the biosynthesis (PHA synthase) and degradation and/or mobilization (intracellular PHA depolymerase) of PHA are located [56–58]. Besides these, other proteins (phasins) thought to be involved in the formation and stabilization of PHA inclusions have also been identified on the surface [59]. Fig. 6 shows a model for the possible structure of in vivo PHA inclusions which is based on the above findings.

2.1.2.1. PHA synthase (PhaC). In the biosynthesis of PHA, the polymerizing enzyme, PHA synthase (PhaC), has been identified as the key enzyme which determines the type of PHA synthesized by the microorganism. To date, almost 40 PHA synthase structural genes from various Gram-positive and Gram-negative bacteria as well as cyanobacteria have been cloned, and the nucleotide sequences from 30 genes have been obtained [21]. Three different types of PHA synthase can be distinguished with respect to their substrate specificities and primary structures [60] (Fig. 4). The three different types of PHA synthase have one strictly conserved cysteine residue which is potentially the active site involved in the polymerization reaction [57].

The first type of PHA synthases, represented by the well-characterized PHA synthase of *R. eutropha*, includes those that are active towards short-chain-length (SCL) HA monomers. The SCL HA monomers are defined as the (*R*)-enantiomers of 3-, 4- and 5-HA containing 3–5 carbon atoms. Very recently it has been shown that small amounts of 3HHx, 3HO and 3HDD units are also incorporated by the PHA synthase of *R. eutropha* [61,62]. Other PHA synthases that apparently prefer SCL HA but can also incorporate 3HHx monomers are those of *A. caviae* [63], *Rhodospirillum rubrum* [64], *Rhodocylus gelatinosus* [65], *Rhodococcus ruber* [28] and *Rhodobacter sphaeroides* [66]. *R. gelatinosus*, like *A. caviae* can also incorporate 3HHp when cultivated on heptanoic acid [65].

The second type of PHA synthases has been characterized as those that efficiently incorporate larger (*R*)-3HA monomers containing 6–14 carbon atoms (termed medium-chain-length [MCL] HA). This type is represented by the two PHA synthases of *P. oleovorans*. It must be noted here that the PHA

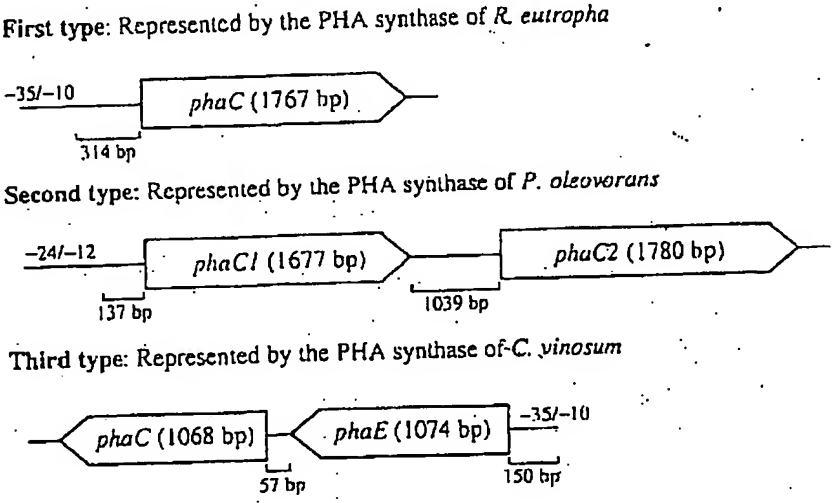


Fig. 4. Classification of PHA synthases (*phaC*) based on their primary structures and substrate specificities.

synthases of *Pseudomonas* species strain 61-3 [67,68] that incorporated MCL HA in their native physiological environment, have been shown to be capable of incorporating significant amounts of SCL HA (3HB units) when the respective genes are expressed in *R. eutropha* PHA-negative mutants.

The third type of PHA synthases has been characterized based on the fact that, unlike the first and second types which consist of only one subunit of about 60–70 kDa, the third type consists of two subunits designated as the C-subunit (~40 kDa) and E-subunit (~40 kDa) [21]. Evidence was obtained to show that both of these subunits are necessary for the functional activity of this group of PHA synthases [60]. This type of PHA synthase has so far been identified in *Chromatium vinosum* [69], *Thiocystis violacea* [70], *Thiocapsa pfennigii* [66] and *Synechocystis* sp. PCC 6803 [71]. The substrate specificities of PHA synthases in this group are apparently not very well defined, but generally they seem to prefer SCL HA [21]. The only exception is the PHA synthase of *T. pfennigii* which is characterized by a broad substrate range comprising both the SCL and MCL HA [66].

PHA synthases do not exhibit significant homologies to other proteins collected in data banks, but it is becoming clearer that they represent a class of highly homologous enzymes based on their primary structures and their substrate specificities [60]. However, their substrate specificities have only been determined in their own native physiological environments, and/or in heterologous physiological environments by genetic engineering techniques. These indirect methods do always give an accurate estimation, but it cannot be overlooked that there are possibilities of limitations posed by the metabolic pathways that supply the monomer units in that particular environment. This has become evident recently due to the differences in the monomer compositions of PHA that are obtained by expression of PHA synthases in various physiological environments. A good example is the revelation of an unusual substrate range for *T. pfennigii* PHA synthase, which only became obvious when it was expressed heterologously in a PHA-negative mutant of *P. putida* [65,72,73].

Detailed studies of the substrate specificities of PHA synthases have for a long time been hampered by difficulties in purifying the enzymes to homogeneity, and also due to limited availability of substrate monomers in the form of coenzyme A thioesters for activity measurements with partially purified enzymes [74]. Recently, the PHA synthase of *R. eutropha* has been successfully purified to 90% homogeneity by overexpression of its gene in *E. coli* [26]. Subsequently, the PHA synthase of *C. vinosum* was also purified [75]. The availability of purified PHA synthases should help to facilitate the detailed examination of their substrate specificities.

2.1.2.2. Intracellular PHA depolymerase (PhaZ). Since PHA is a storage compound for excess carbon, it is natural that microorganisms are equipped with a depolymerizing system to recover the stored carbon. Fig. 5 shows a general scheme for the cyclic metabolism of P(3HB) which has been investigated and reviewed in detail by several research groups [16,19,20]. It should be mentioned here that some microorganisms also produce an extracellular PHA depolymerase which is secreted to degrade crystalline PHA material in the environment. A detailed description about this latter class of depolymerase is given in Section 3.2 of this review. In contrast to PHA synthase, the intracellular PHA depolymerase is relatively not well characterized.

Early studies on the enzymatic depolymerization of PHA inclusions involved the utilization of soluble enzymes from *R. rubrum* to degrade native P(3HB) inclusions isolated from *B. megaterium* KM [56]. The soluble fraction from *R. rubrum* contained a thermostable activator and a thermolabile depolymerase whose activity could be increased by the addition of small amounts of trypsin. In *Zoogloea ramigera* I-16-M, P(3HB) depolymerase activity was found in the soluble enzyme fraction when assayed with

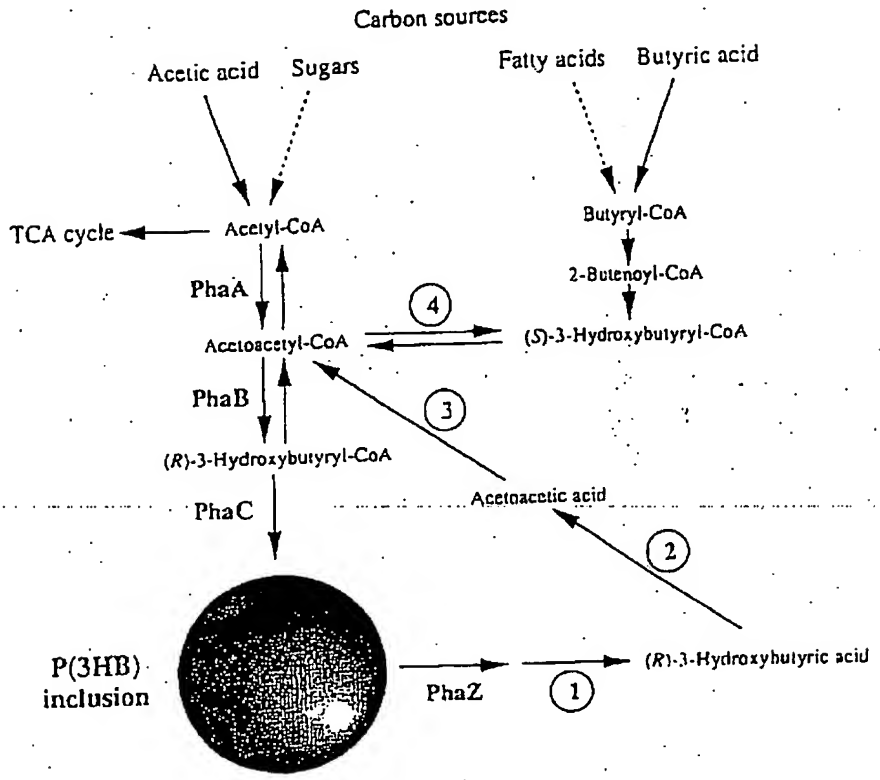


Fig. 5. Cyclic metabolic nature of P(3HB) biosynthesis and degradation in bacteria. PhaA, β-ketothiolase; PhaB, NADPH-dependent acetoacetyl-CoA reductase; PhaC, PHA synthase; PhaZ, PHA depolymerase; 1, dimer hydrolase; 2, (*R*)-3-hydroxybutyrate dehydrogenase; 3, acetoacetyl-CoA synthetase; 4, NADH-dependent acetoacetyl-CoA reductase.

protease-treated P(3HB) inclusions. This led to the suggestion that PHA inclusions may be protected by proteins on the surface from attack by the depolymerase [76]. A recent study proposed that an activator acts to modify an inhibitor present on native P(3HB) inclusions, thereby allowing the intracellular depolymerase access its substrate [77].

Studies using *R. eutropha*, showed that the intracellular degradation of P(3HB) inclusions is a very slow process [78]. The rate of P(3HB) degradation was calculated to be about 10 times slower than the rate of its synthesis [79]. Based on the finding that the number of polymer chains was almost constant during the P(3HB) degradation process, it was suggested that the intracellular depolymerase is an *exo*-type hydrolase acting at the carbonyl terminus of the polymer chain [79]. Low concentrations of diisopropyl fluorophosphate were found to inhibit the depolymerase activity leading to the suggestion that this enzyme is a serine esterase [80]. In comparison to the extracellular depolymerase which attacks crystalline PHA, the mechanism for intracellular depolymerase is presumed to be different because of the amorphous nature of the intracellular PHA inclusions. Nevertheless, the characteristics of lipases and esterases such as the presence of lipase boxes (G X S X G) in the amino acid sequence are thought to be preserved in both types of depolymerase. Based on this, it was assumed that the protein encoded by the *phaZ* genes of *P. oleovorans* and *P. aeruginosa* are probably an intracellular depolymerase [59].

However, concrete evidence for this and the mechanism of intracellular PHA depolymerase are yet to be established.

2.1.2.3. Phasins (PhaP). The close association of proteins on the surface of PHA inclusions have been proposed based on studies done more than 30 years ago [36]. P(3HB) inclusions of *B. megaterium* are composed of 97.7% P(3HB), 1.87% protein and 0.46% lipid [57]. It has been confirmed that the PHA inclusions consist of a hydrophobic core of amorphous PHA [38] that is surrounded by a phospholipid monolayer membrane consisting of various catabolic and non-catabolic proteins (Fig. 6). The catabolic proteins include the PHA synthase and the intracellular PHA depolymerase described above, while the non-catabolic proteins include a group of proteins designated as phasins (PhaP) [59]. The term phasin (*PHA-oleosin*) was coined in analogy to a class of amphiphatic proteins called oleosins [59]. The latter forms close layers at the surfaces of triacylglycerol inclusions within the seeds and pollen of plants. Oleosins were demonstrated to form a boundary layer between the hydrophobic triacylglycerol inclusions and the hydrophilic cytoplasm [81,82]. By extending the surface architecture of triacylglycerol inclusions to PHA inclusions, phasins were presumed to be involved in the stabilization of the amorphous hydrophobic PHA inclusion in the hydrophilic cell cytoplasm.

Interestingly, the genes coding for phasins have been found to be located in the same locus as other PHA biosynthesis genes in some bacteria [83,84]. The phasin protein of *R. ruber* was cloned [85] and two short hydrophobic stretches were identified close to the C-terminus of the protein. These regions have been suggested to be responsible for the binding of the protein to the PHA inclusion [86]. These results strongly suggest that phasins are involved in the in vivo formation of PHA inclusions. A survey on various bacteria demonstrated that phasins are widespread if not essential in PHA-accumulating bacteria [87]. Despite the widespread occurrences of phasins, these proteins are apparently not phylogenetically related, unlike the analogous oleosins, which share a common ancestor [88]. The exact functions of phasins are therefore not very clear but they have been shown to affect the size and production of PHA in some recombinant bacteria [89]. A very recent study has shown that the size of

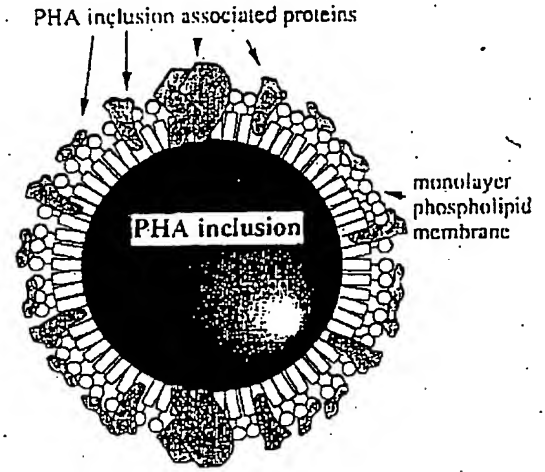


Fig. 6. A model attempting to show the structure of in vivo PHA inclusions and its association with specific proteins. (Not drawn according to actual scale).

PHA inclusions is also affected by the activity levels of enzymes involved in PHA biosynthesis [90]. To this end, higher PHA synthase activities generally seems to result in increased numbers of PHA inclusions, and similar observations have also been made for increased levels of phasin proteins.

2.2. *Physiology of PHA metabolism*

2.2.1. *Conditions favoring PHA accumulation*

The effect of the growth conditions on PHA metabolism was first studied by Macrae and Wilkinson in 1958 for an asporogenous strain of *B. megaterium* [6]. They made the important observation that the quantity of PHA accumulated increased as the carbon to nitrogen ratio increased. Their results suggested that, like polyphosphate and carbohydrate reserves, PHA accumulation occurred in response to an imbalance in growth brought about by nutrient limitations. This significant observation began the investigation into the physiological role of PHA. It was understood later that bacteria make and store PHA when they lack the complete range of nutrients required for cell division but have generous supplies of carbon. The biosynthesis of PHA was shown to be initiated by a magnesium or sulfate deficiency, as well as by nitrogen, phosphate [9,91] and/or oxygen limitations [92].

Physiologically, PHA was first associated with the sporulation of bacteria [93]. It was generally observed that PHA is formed before the onset of sporulation, and the rapid utilization of the polymer precedes sporulation. However, PHA is not always associated with sporulation since not all spore formers make the polymer. The polymer, if present, was therefore thought to be a ready source of carbon and energy for the energy-demanding process of sporulation [94]. To date it is known that PHA is synthesized by a wide range of microorganisms. For many bacteria, the polymer, once accumulated, serves as both carbon and energy source during starvation. PHA constitutes an ideal carbon-energy storage material due to its low solubility and high molecular weight, which exerts negligible osmotic pressure to the bacterial cell [9]. It must be noted that there are also examples of bacteria such as *Alcaligenes latus* [95] and a mutant strain of *Azotobacter vinelandii* [96] which are known to accumulate PHA during growth in the absence of nutrient limitation. However, nitrogen limitation was shown to further enhance the production of PHA in *A. latus* suggesting that this growth during PHA accumulation is not an efficient process [97].

Senior and Dawes [98] proposed that PHA also serves as a sink for reducing power and could therefore be regarded as a redox regulator within the cell. It was found that for members of the Azotobacteriaceae, the reductive step of PHA synthesis appeared to serve as an electron sink for the reducing power which accumulated when electron flow through the electron transfer chain was affected as a consequence of oxygen limitation [99]. On the other hand, in the symbiotic nitrogen fixation process, PHA accumulation has been implicated as both an energy source as well as to serve as a regulatory role, controlling the availability of reducing power for the operation of nitrogenase [100].

2.2.2. *Metabolic pathways leading to the biosynthesis of PHA from various carbon sources*

Since the findings of other components besides 3HB in PHA more than two decades ago, we now know that the PHA synthase enzyme shows a broad substrate specificity and therefore a wide variety of monomers can be polymerized. One of the factors that determines the type of PHA constituents is the carbon source (Fig. 7). Microorganisms are capable of producing PHA from various carbon sources ranging from inexpensive, complex waste effluents like beet/cane molasses [101,102], to plant oils [103] and its fatty acids [104–106], alkanes [13] as well as simple carbohydrates. Based on the types of

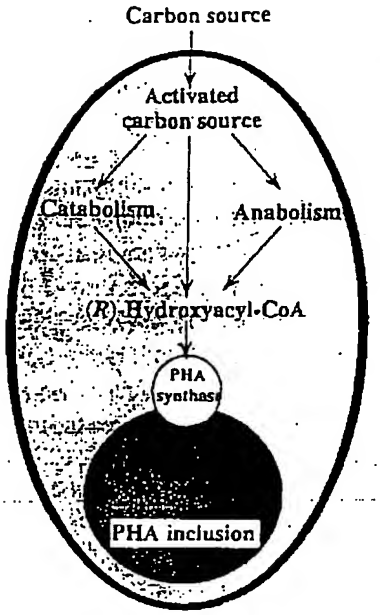


Fig. 7. General scheme for the metabolic pathways of PHA synthesis from different carbon sources within a bacterial cell.

monomer incorporated into PHA, various metabolic pathways have been shown to be involved in the generation of these monomers. Excellent compilations of all of these pathways are available elsewhere [20,107]. The PHA biosynthesis of bacteria can apparently be divided into two major types based on the monomer composition of PHA produced by various wild-type bacteria. *R. eutropha* represents one group while the pseudomonads represent the other major type of PHA biosynthesis. Fig. 8 summarizes the various metabolic pathways that are known to supply monomer units for PHA biosynthesis.

2.2.2.1. PHA biosynthesis represented by R. eutropha. *R. eutropha* is among the bacteria that have been extensively studied for the production of PHA. In *R. eutropha* [19], two acetyl-CoA moieties are condensed to acetoacetyl-CoA by a β-ketothiolase (PhaA). The product then undergoes reduction by an NADPH-dependent reductase (PhaB) which produces the (*R*)-isomer of 3-hydroxybutyryl-CoA (Fig. 8, Pathway I). On the other hand, in *R. rubrum* which shares almost similar PHA biosynthesis pathway as *R. eutropha*, the reductase which is an NADH-dependent isoenzyme, gives rise to the (*S*)-isomer of 3-hydroxybutyryl-CoA. Two enoyl-CoA hydratases [108] then covert the (*S*)-type to the (*R*)-type isomer which is the only stereoisomer usually accepted by the polymerizing enzyme, PHA synthase. The recent genetic analysis of PHA biosynthesis pathways of *A. caviae* had clearly demonstrated the involvement of an (*R*)-specific enoyl-CoA hydratase (PhaJ) in the production of P(3HB-*co*-3HHx) [63,109].

Various carbon sources can be utilized by *R. eutropha* for growth and/or PHA production. Linko and coworkers [110] reported the successful utilization of lactic acid for the production of P(3HB), while it is also known that plant oils are an excellent substrate [103]. *R. eutropha* was also capable of producing the P(3HB) homopolymer from even carbon numbered *n*-alkanoates, while odd-carbon numbered *n*-alkanoates resulted in the accumulation of copolymers of 3HB and 3HV [111]. Studies of PHA production under autotrophic conditions have resulted in the finding that *R. eutropha* can also utilize carbon dioxide

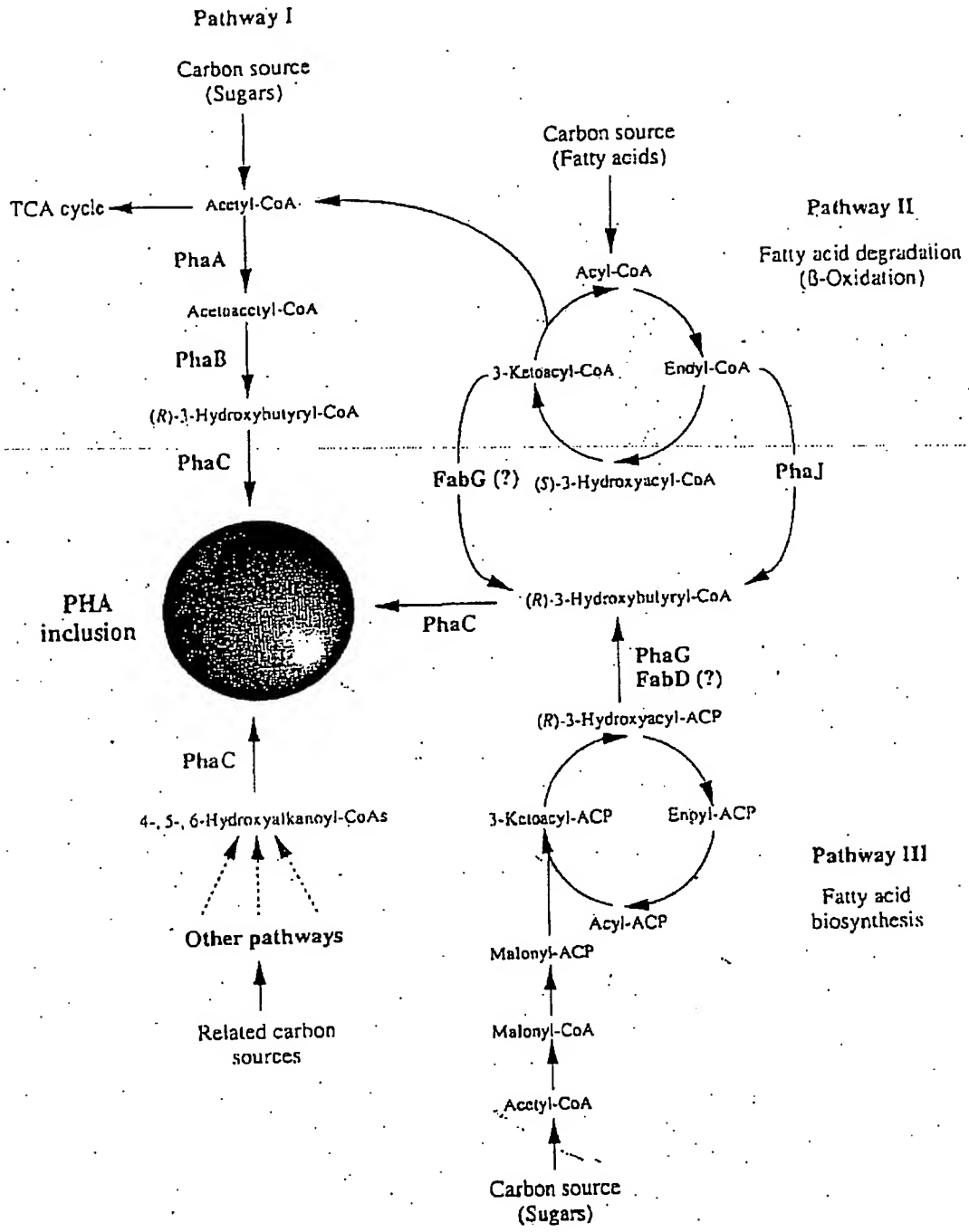


Fig. 8. Metabolic pathways that supply hydroxyalkanoate monomers for PHA biosynthesis. PhaA, β-ketothiolase; PhaB, NADPH-dependent acetoacetyl-CoA reductase; PhaC, PHA synthase; PhaG, 3-hydroxyacyl-ACP-CoA transferase; PhaJ, (*R*)-enoyl-CoA hydratase; FabD, malonyl-CoA-ACP transacylase; FabG, 3-ketoacyl-CoA reductase.

for the production of P(3HB) [112]. In addition to the carbon sources mentioned above, *R. eutropha* is also capable of accumulating PHA from specialized carbon sources such as 4-hydroxybutyric acid, γ-butyrolactone and 1,4-butanediol which gave rise to the incorporation of 4HB monomers along with 3HB [113,114].

Based on the various types of PHA that were synthesized by *R. eutropha*; one common observation is that the incorporated monomers always contain only 3–5 carbon atoms. This lead to the conclusion that in *R. eutropha*, the PHA synthase enzyme which polymerizes the monomers is only active towards SCL HA. However, the position of the oxidized carbon in the monomer is apparently not a crucial factor, which explains the incorporation of 4- and 5-HA besides the more common 3-HA [18]. Very recent findings indicate that the polymerizing enzyme of *R. eutropha* may actually have a broader range of substrate specificity. This was realized when the PHA synthase gene of *R. eutropha* was expressed in a heterologous environment which can provide for a wider range of HA monomers. At present, it has been shown that the *R. eutropha* PHA synthase enzyme can incorporate small amounts of 3HHx, 3HO and 3HDD units [61,62].

2.2.2.2. *PHA biosynthesis represented by the pseudomonads.* Another type of PHA biosynthesis pathway is exhibited by the pseudomonads belonging to the rRNA-homology-group I which can synthesize PHA_{MCL} from various alkanes, alkanols or alkanoates [13]. In contrast to *R. eutropha*, most fluorescent pseudomonads belonging to rRNA homology-group I generally do not synthesize PHA containing SCL monomers (PHA_{SCL}). The range of monomers incorporated into PHA synthesized by these pseudomonads are however much wider [115,116]. Bacteria in this group, derives the 3-hydroxyacyl-CoA substrates of C6–C14 for PHA_{MCL} synthase, from the intermediates of fatty acid β-oxidation pathway (Fig. 8; Pathway II). When *P. oleovorans*, was cultivated on *n*-alkane under nitrogen limitation, the monomer composition of the PHA was found to be a reflection of the substrates that were provided [13]. The largest monomer found in PHA, always contained as many carbon atoms as did the *n*-alkane used as a substrate. For C-even substrates, only C-even monomers were found, the smallest was 3HHx. For C-odd substrates, only C-odd monomers were found, with 3HHp being the smallest monomer [115]. Unsaturated monomers were also incorporated when the carbon sources were 1-alkenes [13]. Specific enzymes such as the enoyl-CoA hydratase (PhaJ) [117] and 3-ketoacyl-CoA reductase (FabG) [118] are presumably involved in the conversion of fatty acid β-oxidation intermediates into suitable monomers that can then be polymerized by the PHA synthase.

Most of the rRNA homology-group I pseudomonads except *P. oleovorans*, can also synthesize PHA containing MCL monomers (PHA_{MCL}) from unrelated carbon sources such as carbohydrates. These bacteria accumulate PHA containing 3-hydroxydecanoate (3HD) as the predominant monomer from various carbon sources such as gluconate, fructose, acetate, glycerol and lactate [119]. In this case, the 3-hydroxyacyl monomers are derived from the de novo fatty acid biosynthesis pathway [119] (Fig. 8, Pathway III). Pseudomonads that have been shown to possess this pathway are *P. aeruginosa*, *P. aureofaciens*, *P. citronellolis*, *P. mendocina* and *P. putida*. Since the fatty acid biosynthesis intermediate is in the form of (*R*)-3-hydroxyacyl-ACP, an additional biosynthetic step is needed to convert it into the (*R*)-3-hydroxyacyl-CoA form. An enzyme known as PhaG, which exhibits a 3-hydroxyacyl-CoA-ACP transferase activity, has been shown to be capable of channeling the intermediates of the de novo fatty acid biosynthesis pathway to PHA biosynthesis [120]. On the other hand, over-expression of transacylating enzymes such as malonyl-CoA-ACP transacylase (FabD) also seems to generate monomers for PHA biosynthesis [121].

'haB.
PhaJ.

Some *Pseudomonas* strains are also capable of accumulating both 3HB and MCL monomers from various carbon sources. Freeze-fracture analysis indicated that both of these polymers were stored in different inclusions in the cells and were therefore in a blend form [122]. Genetic analysis finally revealed that these *Pseudomonas* strains contained at least two different polymerizing enzymes with different substrate specificities [67,123].

To this end, pseudomonads offer the synthesis of a wide variety of optically active (*R*)-HA monomers with functional groups that can be subjected to further chemical modifications [124]. *P. oleovorans* for example, has the ability to incorporate significant proportions of branched-chain HA when appropriate carbon sources are provided as cosubstrates [125]. The functional groups can be potential targets for the subsequent attachment of active compounds, such as drugs for medical treatment [126,127].

2.2.2.3. Other metabolic pathways. Aside from the common 3-HA which is oxidized at the third carbon, other HAs oxidized at a different carbon have also been frequently reported, such as 4-HA. Besides 4-hydroxybutyrate (4HB) [17,114], 4-hydroxyvalerate (4HV) [128], 4-hydroxyhexanoate (4HHx) [72], 4-hydroxyheptanoate (4HHp) and 4-hydroxyoctanoate (4HO) [129] are also known to be constituents of PHA. Other known non-3-HAs are 5-HA [18] and 6-HA [105]. Among these non-3-HAs, the incorporation of 4HB as a monomer into PHA has resulted in the production of commercially attractive copolymers [130,131]. The known wild-type bacteria having the ability to incorporate 4HB into PHA are *R. eutropha* [17,132], *A. latus* [133], *C. acidovorans* [130], *R. ruber* [28], *C. testosteronii* [134] and *Hydrogenophaga pseudoflava* [135]. The bacteria from the genus *Comamonas* apparently have the most efficient pathway to supply 4HB monomers, based on the exceptionally high mole fractions of 4HB (above 90%) in the accumulated PHA copolymers [130,134]. However, HA monomers oxidized at the fourth carbon are usually only derived from related carbon sources such as 4-hydroxybutyric acid, 1,4-butanediol and γ-butyrolactone by the wild-type bacteria.

2.3. Other important features of PHA biosynthesis

2.3.1. Mechanism of PHA synthase

The results of studies in which PHA synthase was incubated with various sulfhydryl inhibitors had suggested that the synthase is a sulfhydryl enzyme [57]. Based on this, the active-site model of PHA synthase was originally proposed by Ballard and coworkers, in which, two thiol groups were suggested to be involved in locating the HA monomers [136]. Kawaguchi and Doi [53] later modified the mechanism suggesting that water may play a role as a chain-transfer agent.

The first successful purification of the PHA synthase of *R. eutropha* enabled detailed biochemical studies which showed that only one thiol group is essential for catalysis [26]. This is supported by the multiple alignment of PHA synthases which shows that only one cysteine residue (Cys-319) is highly conserved that is capable of providing the thiol group [21]. Nevertheless, the most probable reaction mechanism for PHA synthase is based on the well-characterized fatty acid synthase [137,138]. This suggested that two thiol groups are necessary, the second thiol was therefore thought to be available following posttranslational modification via a phosphopantethine moiety [26]. A possible candidate for this modification is the conserved serine residue at position 260 in the PHA synthase structural gene of *R. eutropha*. Recent findings however have failed to support this hypothesis for a specific posttranslational modification [21]. The proposed step involving chain-transfer [53] on the other hand, was contradicted based on the assumption that the PHA synthase is not capable of transferring to a new

chain [139,140]. But analysis using the purified *C. vinosum* PHA synthase suggested a mechanism involving a chain-transfer step [75].

Based on all of the above findings, and also on the observation that purified *R. eutropha* PHA synthase exhibited a significant lag phase [26,139–141], (while this was not observed in the purified PHA synthase of *C. vinosum* [75] which consists of two subunits), a new model for the active PHA synthase has been suggested. The current model suggests that the PHA synthase of *R. eutropha* and other PHA synthases consisting of only one subunit, undergo dimerization to form a homodimer, which would be in agreement with the observed lag phase. This is favorable because the two thiols would then be available from Cys-319 of each PHA synthase. On the other hand, the PHA synthases consisting of two subunits would form a heterodimer whereby the second thiol is speculated to be provided by a conserved Cys-130 of PhaE subunit from *C. vinosum*. This new working model for the active PHA synthase is thus in agreement with the earlier concept of two required thiols for activity.

2.3.2. Factors that affect the molecular weights of PHA

Detailed investigations on the molecular weight of PHA accumulated by bacteria have been scarce despite the fact that this is an important characteristic which could determine applications as a commodity material. This maybe due to the fact that substantial quantities of polymer usually need to be purified from bacterial cells for molecular weight analysis. Another possible reason is that the Mark–Houwink–Sakurada parameters relating the intrinsic viscosity to the molecular weight have only been reported for the P(3HB) homopolymer. Therefore, it is not possible to determine the true molecular weight of PHA containing other monomers. Nevertheless, for comparison purposes the molecular weight data for PHA samples are usually obtained by gel permeation chromatography (GPC) analysis where polystyrene samples with low polydispersities are used as standards to construct a calibration curve [16].

A wide range of molecular weights are exhibited by PHA from different microorganisms and also from different stages and conditions of cultivation. The largest reported M_n value for biologically synthesized PHA is about 20 MDa [30]. In the same report, it was also shown that the pH of the culture medium can greatly affect the M_n of the PHA produced. Also, the type and concentration of the carbon source supplied can affect the M_n of the PHA produced [142].

The controlled expression of the *R. eutropha* PHA synthase gene in recombinant *E. coli* showed that an increase in the activity of PHA synthase resulted in a decrease in the M_n of the PHA produced [143]. Similar observations were also previously made in *Pseudomonas* strains when the copy number of its PHA synthase genes was increased [144]. These observations indicated that the molecular weight of PHA is determined by the activity of the PHA synthase [107]. In *C. acidovorans*, a significant increase in the activity of PHA synthase was achieved by increasing the copy number of its PHA synthase gene, which resulted in an improved production of P(4HB) from 4-hydroxybutyric acid [145]. Molecular weight analysis of the P(4HB) produced by the recombinant strain however did not show any significant differences from that produced by the wild type *C. acidovorans*. The molecular weight of P(3HB) produced by recombinant *R. eutropha* was also found to be independent of the levels of PHA synthase activity [146]. Based on these contradicting results, it is difficult to draw a conclusive correlation between PHA synthase activity and the molecular weight of PHA produced in vivo.

2.3.3. The occurrences of low molecular weight PHA

Besides functioning as a carbon and energy storage compound, other possible functions of PHA have been gaining interest recently. Studies on the ability of a microbial cell to take up genetic material from

an external medium (known as competence) have led to the identification of another type of PHA which is a constituent of cytoplasm [147], and cytoplasm membranes [148]. In this case however, the PHA consisted of low molecular weight P(3HB) of about 13,000 Da (corresponding to ca 150 monomers). Further studies established a relationship between competence and the P(3HB) content in *E. coli* cells which has led to the postulation of a P(3HB)/calcium polyphosphate channel within the bacterial cytoplasm membrane [149].

It is increasingly convincing that PHA, especially P(3HB), is not just an inert storage polymer confined to certain bacteria, but is instead a ubiquitous, interactive, solvating biopolymer involved in important physiological functions. This low molecular weight non-storage PHA referred to as cP(3HB) ("c" for complexed) is associated to other macromolecules and is widely distributed in biological cells, being found in representative organisms of nearly all phyla [150,151]. It would be interesting to study how cP(3HB) is synthesized and to determine how the molecular weight is regulated. It is possible that other enzyme(s) may also be present which is (are) capable of polymerizing 3HB monomers into low molecular weight PHA.

2.4. Manipulation of PHA biosynthesis by genetic engineering techniques

Ever since the entire three-gene set that directs P(3HB) biosynthesis in *R. eutropha* was cloned [22], the technology has been available to express these genes in different organisms. In *R. eutropha*, the PHA biosynthetic genes were found to be clustered in one operon in the order *phaCAB*, i.e. the genes coding for PHA synthase, β-ketothiolase and acetoacetyl-CoA reductase. Since then, many other operons and genes involved in PHA biosynthesis have been identified and characterized from various microorganisms [21]. *E. coli* has been largely used as the model system to further study the heterologous expression of these genes and for the purpose of engineering novel metabolic pathways.

2.4.1. Recombinant E. coli as a producer of PHA

The physiology, biochemistry and genetics of *E. coli* have been studied in great detail. This makes *E. coli* the perfect host as an heterologous expression background for various foreign proteins. The biosynthesis of PHA can be easily achieved in *E. coli* by introducing the *phaCAB* operon of *R. eutropha* [22,23,25]. *E. coli* is considered a better commercial producer of PHA because it can use a wider range of cheap carbon sources, and also because it is easier and less costly to purify the polymer from this bacteria [152]. Besides that, since *E. coli* do not have an intracellular PHA depolymerase (because *E. coli* is not a natural PHA producer), the synthesized PHA will not be degraded. This is probably one of the reasons that had enabled the production of very high molecular weight PHA using recombinant *E. coli* [30]. It has also been shown that the molecular weight can be controlled by modulating the activity of PHA synthase [143]. On the other hand, by using specific mutants of *E. coli*, recombinant strains have also been constructed which can produce for example a copolymer of P(3HB-*co*-3HV) [153]. Employing a β-oxidation mutant of *E. coli* LS1298 (*fadB*), the expression of the PHA synthase gene from *P. aeruginosa* had enabled the production of PHA_{MCL}, indicating that the β-oxidation pathway in *E. coli* provides HA monomers for PHA biosynthesis [154,155]. In other cases, the expression of the PHA synthase gene from *A. caviae* along with *phaJ* or *fabG* genes was shown to direct the synthesis of P(3HB-*co*-3HHx) copolymers in *E. coli* [117,118]. Besides that, it has also been shown that a non-3-HA, such as 4HB-containing copolymers and homopolymer, can also be synthesized by recombinant *E. coli* cultivated on either glucose [156] or on glucose plus 4-hydroxybutyric acid [157], respectively.

In summary, *E. coli* offers a well-defined physiological environment for the construction and manipulation of various metabolic pathways to produce a wide range of PHA from cost effective carbon sources.

2.4.2. Transgenic plants as potential producers of PHA

Cost effectiveness is one of the main factors that has hampered the usage of PHA as a commodity plastic. In that sense, the ultimate cost effective producer of PHA would of course be transgenic plants [158,159]. Several initial attempts using transgenic *Arabidopsis thaliana* were successful in producing small amounts of P(3HB). However, the host plants were adversely affected probably due to depletion of one or more essential substrates for growth [27]. This problem was then eliminated by genetic manipulations and P(3HB) was more effectively produced in the transgenic plant plastids [160]. There was also an attempt to produce P(3HB) within cotton fiber lumen, thus modifying the chemical and thermal properties of fiber. It was shown that transgenic cotton plants (*Gossypium hirsutum* L. cv DP50) can be directed to produce P(3HB) in cotton fiber cells [161]. Very recently, the construction of transgenic *A. thaliana* producing PHA_{MCL} was also successfully achieved by using the PHA synthase gene of *P. aeruginosa* [162]. This achievement indicates that the β-oxidation of plant fatty acids can generate a broad range of saturated and unsaturated (*R*)-3-hydroxyalkanoate monomers that can be used to synthesize PHA_{MCL}. The successful generation of metabolic precursors for the production of the commercially attractive P(3HB-*co*-3HV) copolymer in plants was recently reported [163]. There are also preliminary evidence to suggest that it may even be possible to generate 4HB monomers in plants to produce a P(3HB-*co*-4HB) copolymer [164].

All of these achievements indicate that it is possible to direct the synthesis of PHA to a specified location in transgenic plants. For large-scale production, it has been suggested that oilseed crops such as rapeseed would appear to be a particularly good target crop since results obtained with PHA production in the closely related species *A. thaliana* could be directly applied to rapeseed [165]. The recent discoveries of metabolic links to PHA from fatty acid biosynthesis [120] and β-oxidation [109] in bacteria will be additional advantages since; in plants the PHA production pathway would eventually have to be metabolically engineered from unrelated carbon sources [159].

2.4.3. Other potential PHA production systems

The production of PHA has also been achieved in insect cells of *Spodoptera frugiperda* with a modified eukaryotic fatty acid synthase [166]. Here it was shown that the dehydrase-domain mutant rat fatty acid synthase cDNA can be used to generate (*R*)-3-hydroxybutyryl-CoA via de novo synthesis. This modified rat fatty acid synthase together with the PHA synthase of *R. eutropha* were used to redesign the PHA biosynthesis pathway in insect cells resulting in successful P(3HB) accumulation [166]. Besides that, another eukaryotic system that has been exploited for the production of PHA is the yeast, *Saccharomyces cerevisiae*: In this case, the introduction of the *R. eutropha* PHA synthase gene alone could direct the accumulation of small amounts PHA in the cytoplasm [167]. In order to increase further the P(3HB) production capacity in *S. cerevisiae*, the monomer supplying pathway apparently needs to be enhanced [107].

The in vitro synthesis of PHA using partially [74,168] or fully [26,75] purified PHA synthases also offers an interesting method of producing PHA. This is promising since an improved method for the expression of PHA synthase from *R. eutropha* was developed using a baculovirus *Autographa californica* nuclear polyhedrosis virus (AcMNPV) in BTI-TN-5B1-4 *Trichoplusia ni* cells which results

in high-level production of active PHA synthase [169]. The availability of purified PHA synthase coupled with an enzyme system like that of the modified rat fatty acid synthase which could provide the necessary precursor molecules maybe an alternative method to synthesize PHA in vitro. Recent reports have, in fact, demonstrated achievements in the in vitro synthesis of P(3HB) by using a three-enzyme system that can recycle the expensive coenzyme A [170,171].

Another potential production system for PHA may be cyanobacteria which are oxygenic photoautotrophic microorganisms [172]. The identification of PHA in cyanobacteria dates back to 1966 [173]. However, most reports were based on ultramorphological evidence only or did not use pure cultures [174]. The prospect of using cyanobacteria to produce PHA is interesting and lately much effort is being directed to this alternative approach [175,176]. Recently the first cloning of PHA synthase from a cyanobacterium was achieved, and the primary structure shows that the enzyme consists of two subunits, i.e PhaC and PhaE [71].

In future, the production of PHA by using transgenic plants is expected to reduce costs to economically acceptable levels [159,165]. Considering the soaring world population and the increased demand for food, fertile land may have to be used efficiently for food production. In such a situation, the utilization of recombinant cyanobacteria and/or microalgae for PHA production may be another option. Large-scale production of *Spirulina* for consumption as dietary and food supplements, animal feed, and also as biofertilizer seems to be feasible [177,178]. An ideal process would be where PHA can be harvested as a byproduct of bioremediation process employing cyanobacteria.

2.5. Potential therapeutic applications for PHA

Among the various types of PHA that have been identified to date, those containing 4-hydroxybutyrate (4HB) monomers may hold promise as a polymer with potential therapeutic value. 4HB (generally termed γ-hydroxybutyrate [GHB] in the medical field) was initially used as an intravenous anaesthetic agent in Europe and Japan because it can cross the blood–brain barrier rapidly to induce a sleep-like state with cardiovascular stability [179,180]. In fact, 4HB was found to be a normal metabolite in extracts of brain tissue of rat, pigeon and man [181]. Later, 4HB was used for the treatment of narcolepsy as it increased slow wave sleep (SWS) and rapid-eye-movement (REM) sleep by inducing the symptoms of narcolepsy and containing them at night [182]. It is worth noting that in the United States, the Food and Drug Administration (FDA) approved the use of 4HB in investigational research such as narcolepsy trials [183]. More recently, the use of 4HB has been advocated in the treatment of alcohol addiction, including alcohol withdrawal syndrome [184,185], following the finding that 4HB increases brain dopamine levels [186]. A number of studies have also indicated that 4HB can reduce energy substrate consumption in both brain and peripheral tissues, which means that it can protect these tissues from the damaging effects of anoxia or excessive metabolic demand [187].

The potential therapeutic value of 4HB was realized as early as the 1960s, as is obvious from the interest shown by pharmaceutical companies [188]. Since 4HB is not available commercially in pure form, its sodium salt has been widely used as an anesthetic [189] and in investigational research where it was administered either orally or intravenously. However, a handy oral application in the form of tablets or capsules was not possible due to the strong hygroscopicity of 4HB salts. Therefore, methods for the preparation of non-hygroscopic salts of 4HB were developed [190]. It must also be noted that 4HB itself is a relatively cheap, naturally occurring compound and therefore it could not be patented [191]. This may be one of the reasons why 4HB has not been aggressively developed as a therapeutic agent, despite

its many benefits when used responsibly [188]. Nevertheless, several patents have been filed describing the use of 4HB as a sleeping drug [190] and in the treatment of alcohol withdrawal syndrome [192]. Methods have also been patented for the controlled-release of 4HB for the treatment of alcoholism as well as heroin and nicotine addiction [193].

It is of interest to note that 4HB, like 3HB [194] is a naturally occurring substance in many organisms [195] and that they are both members of the family of bacterial PHA [17]. P(3HB-*co*-4HB) is a biodegradable and biocompatible copolymer, and therefore it may have potential application in the controlled release of 4HB for therapeutic purposes. A patent describing the application of 4HB-containing PHA as a slow-releasing system for biomedical purposes was filed more than 10 years ago [196]. Subsequently, the hydrolytic and enzymatic degradation processes of P(3HB-*co*-4HB) films in vitro was studied by monitoring the time-dependent changes in molecular weights and weight loss (erosion) [114,197]. It is worth noting that the film of P(3HB) homopolymer was not attacked by the lipase from *Rhizopus delemer*, but it resulted in a significant erosion rate for the P(4HB) film [198]. Further studies showed that the erosion rate of PHA films by lipase increased with an increase in the 4HB fraction. P(3HB-*co*-4HB) films were shown to be hydrolyzed by both PHA depolymerase and lipase [130].

While there are many studies on the in vitro degradation of P(3HB-*co*-4HB) films, apparently only one report is available on its in vivo degradation in rats. In the latter study, P(3HB-*co*-10 mol% 4HB) films were implanted into the intraperitoneal area of rat stomach and changes in molecular weights (M_w and M_n) were monitored over a period of 4 months. A 20% decrease in the value of M_n was observed with no notable signs of cytotoxicity in the implantation area [199]. This result shows that P(3HB-*co*-4HB) implantation does result in some sort of degradation in vivo, which may be due to the action of lipases since lipases from several sources have been shown to be capable of hydrolyzing P(4HB) films [200]. However, it is not known if the degradation products consist of 4HB monomers in a pharmacologically active form since the above-mentioned study did not include detailed clinical analysis. Therefore no conclusion can be made relating to the pharmacological effects of P(3HB-*co*-4HB) degradation products on the rat subjects.

The possible applications of 4HB-containing PHA to deliver 4HB for therapeutic purposes should be carefully examined. It has been shown that high molecular weight P(3HB-*co*-4HB) copolymers containing 0–100 mol% 4HB can be easily produced and that the degradation rate can also be controlled [130,131]. It may also be possible to prepare co- and terpolymers of PHA containing 4HB and other HA monomers which might show different rates of degradation and modes for releasing 4HB. PHA copolymers containing random/block distributions of 4HB can also be synthesized by controlling the feeding regimen of carbon sources to the bacteria. Thus, it might be possible to precisely control the release of 4HB in a predetermined dosage over a short/long period of time. Much clinical work needs to be performed before the potential therapeutic value of 4HB-containing PHA can be harnessed.

3. Part II: physicochemical aspects of PHA

3.1. Structure and properties of PHA

3.1.1. Poly[(R)-3-hydroxybutyrate] homopolymer

It has been mentioned earlier that P(3HB) is the most common biological polyester produced by

various microorganisms in nature [9,19]. It is also clear that this polyester has a perfctly isotactic structure with only the (*R*)-configuration. P(3HB) isolated from bacteria possesses 55–80% crystallinity [201], while the molecules within bacteria are amorphous [38,39,202] and exist as water insoluble inclusions.

3.1.1.1. Crystalline structure of poly[(R)-3-hydroxybutyrate]. The crystal structure of P(3HB) has been determined by X-ray studies on oriented fibers [203,204]. The fiber diagram indicated a repeat along the chain axis of 0.596 nm, corresponding to the length of two residues with two antiparallel chains packed in an orthorhombic unit cell with dimensions $a = 0.576$ nm, $b = 1.320$ nm and c (fiber axis) $= 0.596$ nm and space group $P2_12_12_1$. Resulting from the conformational analysis based on intramolecular energy calculations, it was revealed that the molecule has a left-handed 2_1-helical conformation [203–206].

P(3HB) single crystals have been prepared from many kinds of solvent [51,207–218], and their surface morphologies and crystal structures were investigated using microscopic analysis. Typically, P(3HB) forms lath-shape crystals with dimensions of around 0.3–2 μm for the short and 5–10 μm for the long axes. Based on an electron diffractogram for a P(3HB) single crystal, the long axis of P(3HB) single crystals is the crystallographic *a*-axis. The thickness of single crystals is in the range of 4–10 nm depending on the molecular weight, solvent and crystallization temperature. Barham et al. [209] have shown that a single crystal of P(3HB) can be split into small crystal fragments along the long-axis direction by stretching the material perpendicular to its long axis. When the single crystal was stretched parallel to its long axis, periodic cracks intersected the long axis. In addition, they performed polyethylene decoration on the surface of P(3HB) single crystals. The decorated polyethylene appeared as crystals and have their long axis perpendicular to the long axis of P(3HB) single crystal [215,218]. These results suggested that the predominant chain folding in P(3HB) crystals is along the long axis of the single crystal, that is, along the [100] direction with existing successive folds in the [110] and [110] directions.

Single crystals with well-defined structures are monolamellar systems. In contrast, bulk materials such as films and plates are usually multilamellar systems that have aggregated into multi-oriented lamellar crystals. P(3HB) chains typically form spherulites when crystallized from the melt in bulk materials [209]. In P(3HB) spherulites, the lamellar crystals of P(3HB) grow radial with stacking. The crystallographic *a* axis is radial with *b* and *c* axes rotating about it. Due to the twisting of P(3HB) lamellar crystals, P(3HB) spherulites typically show banded textures. The periodicity and regularity of the banding texture depend both on the crystallization temperature and on the molecular weight. The kinetics for the growth rate of P(3HB) spherulites have been evaluated at various crystallization temperatures, and the growth rate shows a maximum value at around 90°C. The overall crystallization rate of P(3HB) shows a maximum in the temperature range of 50–60°C.

3.1.1.2. Physical properties of poly[(R)-3-hydroxybutyrate]. The M_w of P(3HB) produced from wild-type bacteria is usually in the range of 1×10^4–3×10^6 g/mol with a polydispersity of around two [16]. The glass transition temperature of P(3HB) is around 4°C while the melting temperature is near 180°C, as measured by calorimetric analysis. The densities of crystalline and amorphous P(3HB) are 1.26 and 1.18 g/cm^3, respectively. Mechanical properties like the Young's modulus (3.5 GPa) and the tensile strength (43 MPa) of P(3HB) material are close to those of isotactic polypropylene. The extension to break (5%) for P(3HB) is however markedly lower than that of polypropylene (400%). Therefore, P(3HB) appears as a stiffer and more brittle plastic material when compared with polypropylene.

Much work has been carried out to understand further the reasons behind the brittle nature of P(3HB) and to improve the polymer's physical properties. When spherulites of P(3HB) homopolymer are grown from the melt, large scale cracks are often visible in the spherulites. Barham and coworkers suggested that the formation of such cracks in P(3HB) spherulites is one of the reasons that gives rise to embrittlement [219–221]. De Koning and Lemstra [222], reported that the embrittlement of P(3HB) materials occurs during storage after their initial crystallization from the melt. The secondary crystallization has been argued to result in the reorganization of lamellar crystals formed during the initial crystallization process, which tightly constrains the amorphous chains between crystals. They demonstrated that P(3HB) materials can be toughened using an annealing treatment after initial crystallization [223].

Advances in the biochemistry and molecular biology of PHA biosynthesis have resulted in the cloning of PHA biosynthesis genes from various bacteria [20,21]. This has allowed for the development of recombinant bacteria which can produce PHA under the direction of heterologous genes [152]. It was recently shown that a recombinant *E. coli* harboring PHA biosynthesis genes from *R. eutropha* can produce ultra-high molecular weight P(3HB) homopolymer. The weight-average molecular weight values were found to be in the range of 3×10^6–1.1×10^7 under special fermentation conditions [224].

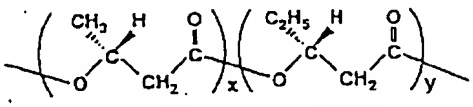
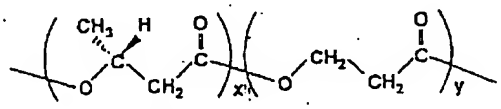
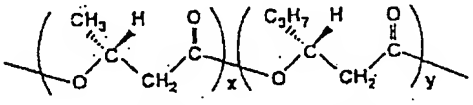
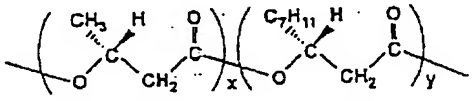
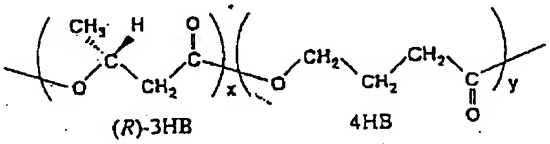
The preparation of P(3HB) stretched films has been successful for the ultra-high molecular weight P(3HB), and it was found that the mechanical properties of the stretched P(3HB) film were markedly improved relative to those of the unstretched film [224]. The elongation break, Young's modulus and tensile strength for stretched films were 58%, 1.1 GPa and 62 MPa, respectively. In addition, when annealing treatment was applied to the stretched film, it was found that the mechanical properties were further improved. The melting temperature of the stretched P(3HB) film with annealing treatment was around 190°C. Thus, P(3HB) homopolymer which was initially a brittle material with poor physical properties, is now, with the help of genetic engineering techniques, a potential candidate for further commercial exploitation.

3.1.2. Copolymers of (R)-3-hydroxybutyrate with hydroxyalkanoates

Besides the improvement in the physical properties of P(3HB) due to increased molecular weight, the incorporation of other HA units to form PHA copolymers can also improve its physical properties. Random copolymers containing (*R*)-3HB as a constituent along with other HA units of chain lengths ranging from 3–14 carbon atoms have been produced from various carbon substrates by a variety of bacteria. As mentioned earlier in Section 2 of this review, the PHA composition produced by bacteria is dependent on the substrate specificities of enzymes in the PHA biosynthesis pathway. Table 2 shows the structures for typical PHA copolymers and their producers.

3.1.2.1. Crystalline structure of PHA copolymers. The structures and properties of random copolymers of (*R*)-3HB and (*R*)-3HV have been investigated most extensively. P(3HB-*co*-3HV) copolymers have approximately the same high degrees of crystallinity (50–70%) throughout a wide range of copolymer compositions [225]. A structural characteristic of P(3HB-*co*-3HV) is isodimorphism, i.e. cocrystallization of the two monomer units in either of the homopolymer crystal lattices of P(3HB) and P(3HV), depending on whether the (*R*)-3HV composition is above or below ~40 mol%. Bluhm et al. [226] first reported that there is significant cocrystallization of the minor unit in the host crystalline lattice. The partitioning of the (*R*)-3HV unit in the P(3HB) crystal lattice has been dealt with both theoretically [227] and experimentally [225,228–233]. From the results of density measurements, X-ray diffraction analysis, thermal measurements and solid-state NMR analysis, the composition of (*R*)-3HV units in the

Table 2
Microbial synthesis of PHA copolymers containing (*R*)-3HB as a constituent

Bacterial strain	Carbon substrate	Random copolymer
Ralstonia eutropha	Propionic acid	 (*R*)-3HB (*R*)-3HV
Ralstonia eutropha	Pentanoic acid 3-Hydroxypropionoc acid	 (*R*)-3HB 3HP
Alcaligenes latus *Aeromonas cavie*	1,5-Pentanediol Plant oils	 (*R*)-3HB (*R*)-3HHx
Pseudomonas sp. *Ralstonia eutropha*	Sugar 4-Hydroxybutyric acid	 (*R*)-3HB (*R*)-3HD
Alcaligenes latus *Comamonas acidovorans*	γ-Butyrolactone 1,4-Butanediol 1,6-Hexanediol	 (*R*)-3HB 4HB

P(3HB) crystalline phase has been determined to be about 2/3 of the overall composition in the range of 0–30 mol% (*R*)-3HV. However, the considerable reductions of both the rate of crystal growth and the heat of fusion suggest that the (*R*)-3HV units have to be regarded as energetically unfavorable defects in P(3HB) crystalline phase.

Single crystals of P(3HB-*co*-3HV) have been reported by Mitomo et al. [210], Marchessault et al. [211], Nobes et al. [234] and Iwata et al. [218,235]. Mitomo et al. obtained P(3HB-*co*-3HV) single crystals with six different (*R*)-3HV contents of less than 30 mol% by isothermal crystallization in

propylene carbonate, and reported that the morphological characteristics of single crystals up to 10 mol% of (*R*)-3HV content were very similar to P(3HB), while single crystals containing 17 and 30 mol% (*R*)-3HV yielded a morphological irregularity with bumpy surfaces. A slight increase in the *a* parameter from 0.576 to 0.581 nm (10 mol% (*R*)-3HV) was also noted [210]. Single crystals of P(3HB-*co*-3HV) with increasing (*R*)-3HV contents (up to 21 mol%) were subsequently grown from ethylene glycol by Marchessault et al., who reported that single crystals exhibited electron diffraction diagrams of the P(3HB) lattice, but with a slight increase in the *a* and *b* parameters [211]. Recently, Iwata et al. prepared single crystals of P(3HB-*co*-8 mol% 3HV) from chloroform/ethanol and reported that the (*R*)-3HV units are excluded from the crystals and exist mainly on the crystal surfaces during slow crystallization, since there is no essential difference in the *d*-spacings for the electron diffraction diagrams between P(3HB) homopolymer and P(3HB-*co*-8 mol% 3HV) single crystals [218,235].

In the case of random copolymers of (*R*)-3HB with (*R*)-3HHx having a propyl side chain, the X-ray crystallinities decreased from 60 to 18% as the (*R*)-3HHx fraction increased from 0 to 25 mol%. The crystallographic parameters of the P(3HB-*co*-3HHx) copolyesters were little influenced by the presence of the (*R*)-3HHx unit, suggesting that the (*R*)-3HHx units are excluded from the P(3HB) crystalline phase. The rates of crystal growth for P(3HB-*co*-3HHx) were markedly reduced with an increase in the (*R*)-3HHx fraction, indicating that the randomly distributed (*R*)-3HHx units in P(3HB-*co*-3HHx) lead to a remarkable decrease in the rate of deposition of the (*R*)-3HB segments at the growing front of P(3HB) crystalline lamellae [236].

Crystal structures and the degrees of crystallinity for P(3HB-*co*-3HP) copolymers have been determined from the X-ray diffractions of solution-cast films having a wide range of compositions of 0–100 mol% 3HP [237,238]. Only one crystalline form for the P(3HB) lattice was observed for copolymers with compositions up to 43 mol% 3HP. The crystallographic parameters of the copolyesters with compositions up to 43 mol% 3HP were little influenced by the presence of the 3HP unit, and the X-ray crystallinities decreased from 60 to 7% as the 3HP fraction was increased from 0 to 67 mol%, suggesting that the 3HP units are also excluded from the P(3HB) crystalline phase. From the measurement of ^{13}C spin-lattice relaxation times using solid-state NMR, it was revealed that the cocrystallization of (*R*)-3HB and 3HP units in the same crystal lattice does not occur in the P(3HB-*co*-3HP) copolymer [239].

The crystallinity of P(3HB-*co*-4HB) decreased from 60 to 14% as the 4HB content increased from 0 to 49 mol% [130,131]. Only one crystalline form of the P(3HB) lattice was observed for the X-ray diffraction patterns of P(3HB-*co*-4HB) copolymers with compositions of 0–29 mol% 4HB. In contrast, only the P(4HB) lattice was observed for the P(3HB-*co*-4HB) copolymers with compositions of 78–100 mol% 4HB. Also, in the case of P(3HB-*co*-4HB), the rates of crystal growth decreased with the 4HB fraction, suggesting that the 4HB units are excluded from the P(3HB) crystalline phase [130,131].

Recently, Iwata et al. have prepared single crystals of three types of P(3HB) copolymer with (*R*)-3HHx, 4HB and 6-hydroxyhexanoate (6HH) units as the second monomer [218,235]. Each single crystal of a copolyester showed a lath-shaped morphology, and yielded a sharp electron diffraction pattern similar to that for the P(3HB) homopolymer. In their study, there was no essential difference in the *d*-spacings for the electron diffraction diagrams between the P(3HB) homopolymer and copolymer single crystals, suggesting that the second monomers are excluded from the crystals and mainly exist on the crystal surfaces.

The lamellar structures of PHA copolymer films crystallized at various temperatures from the melt have been characterized by small-angle X-ray scattering [240]. The lamellar core thickness of the

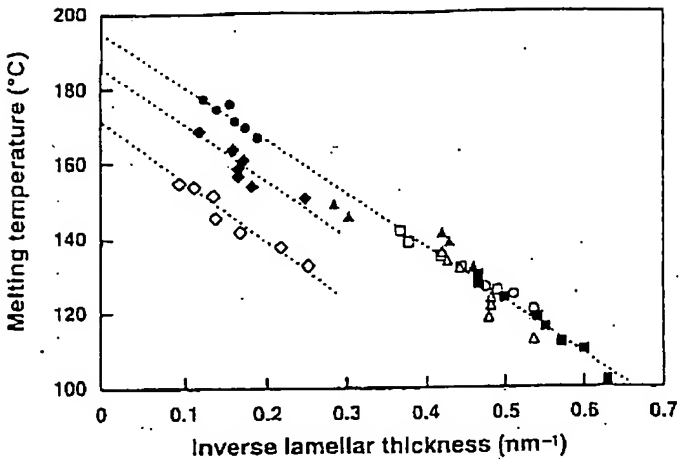


Fig. 9. Variation in melting temperature (T_m) as a function of the inverse lamellar thickness ($1/l_c$) for melt-crystallized polyester films: (●), P(3HB); (◆), P(3HB-*co*-6 mol% 3HV); (◇), P(3HB-*co*-16 mol% 3HV); (○), P(3HB-*co*-8 mol% 3HHx); (▲), P(3HB-*co*-8 mol% 4HB); (△), P(3HB-*co*-10 mol% 4HB); (■), P(3HB-*co*-10 mol% 6HH); and (□), P(3HB-*co*-5 mol% LA).

P(3HB) homopolymer increased from 5.3 to 8.2 nm, as the crystallization temperature was increased from 60 to 130°C. The lamellar core thickness (1.6–3.5 nm) of the copolyester samples (except for the P(3HB-*co*-3HV) copolymers) were smaller than those for the P(3HB) homopolymer samples. In contrast, the lamellar thickness (4.0–10.8 nm) of the P(3HB-*co*-3HV) copolymers was almost consistent with the value of the P(3HB) homopolymer at a given crystallization temperature.

Fig. 9 shows the relationship between the inverse lamellar thickness ($1/l_c$) and the melting temperature (T_m) for melt-crystallized polyester samples. For the P(3HB) homopolymer, the plot of melting temperature against inverse lamellar thickness shows a linear relationship, and the equilibrium melting temperature (T_m^0) can be determined from the intercept of the line as 194 ± 6°C. For the P(3HB-*co*-3HV) samples, the lines shifted to lower melting temperatures at the same lamellar thickness with an increase in the (*R*)-3HV fractions. The plots of T_m against $1/l_c$ for the other PHA copolyester samples fitted with the line obtained from plotting the T_m and $1/l_c$ for the P(3HB) homopolymer samples extrapolating toward thinner lamellar thickness. This result indicates that the crystalline structure of lamellae for copolyesters, except for P(3HB-*co*-3HV), is essentially the same as that for the P(3HB) homopolymer. It has been concluded that the randomly distributed second monomer units (except for (*R*)-3HV in P(3HB) copolyesters) act as defects in the P(3HB) crystal and are excluded from the P(3HB) crystalline lamellae.

In the melting endotherms of melt-crystallized films for PHA copolymers, both a broad peak starting around room temperature and a sharper peak starting above the isothermal crystallization temperature were observed [240,241]. The microscopic analysis of lamellar crystals in relatively thin layers (around 100 nm thickness) of copolyester revealed the formation of thin crystals with 1–4 nm thickness during storage at room temperature after primary crystallization. This result suggested that long sequences of (*R*)-3HB units in a random copolyester form relatively thick P(3HB) crystalline lamellae during the primary crystallization process at a given crystallization temperature, while that shorter sequences of (*R*)-3HB units, which are incapable of crystallizing at a given crystallization temperature, form relatively thin crystalline lamellae during the subsequent crystallization process at room temperature [241].

3.1.2.2. Physical properties of PHA copolymers. The physical and thermal properties of PHA copolymers can be regulated by varying their molecular structures and copolymer compositions. The P(3HB) homopolymer is a relatively stiff and brittle material. The introduction of HA comonomers into a P(3HB) chain greatly improves its mechanical properties [16,201]. The PHA family of polyesters offers a wide variety of polymeric materials exhibiting various properties, from hard crystalline plastics to elastic rubbers. The PHA materials behave as thermoplastics with melting temperatures of 50–180°C.

For solution-cast films of P(3HB-*co*-3HHx) copolymer, the melting temperature decreased from 177 to 52°C as the (*R*)-3HHx fraction was increased from 0 to 25 mol%. The glass-transition temperature decreased from 4 to −4°C, as shown in Fig. 10. The tensile strength of the films decreased from 43 to 20 MPa as the (*R*)-3HHx fraction was increased from 0 to 17 mol%. In contrast, elongation to break increased from 6 to 850%. Thus, the P(3HB-*co*-3HHx) films become soft and flexible with an increase in the (*R*)-3HHx fraction [236].

For solution-cast films of the P(3HB-*co*-3HP) copolymer, the melting temperature decreased from 177 to 44°C with the 3HP fraction and then increased to 77°C as the 3HP fraction was increased from 67 to 100 mol%. The glass-transition temperature decreased from 4 to −19°C as the 3HP fraction was increased from 0 to 100 mol% [237,238].

The melting temperatures for P(3HB-*co*-4HB) solution-cast films decreased from 178 to 130°C with the 4HB fraction. The glass-transition temperature decreased from 4 to −48°C as the 4HB fraction was increased from 0 to 100 mol%. The tensile strengths of P(3HB-*co*-4HB) films with compositions of 0–16 mol% 4HB decreased from 43 to 26 MPa with an increase in the 4HB fraction, while elongation to break increased from 5 to 444%. The tensile strength of films with compositions of 64–100 mol% 4HB increased from 17 to 104 MPa with increasing 4HB fractions. The true tensile strength of P(4HB)

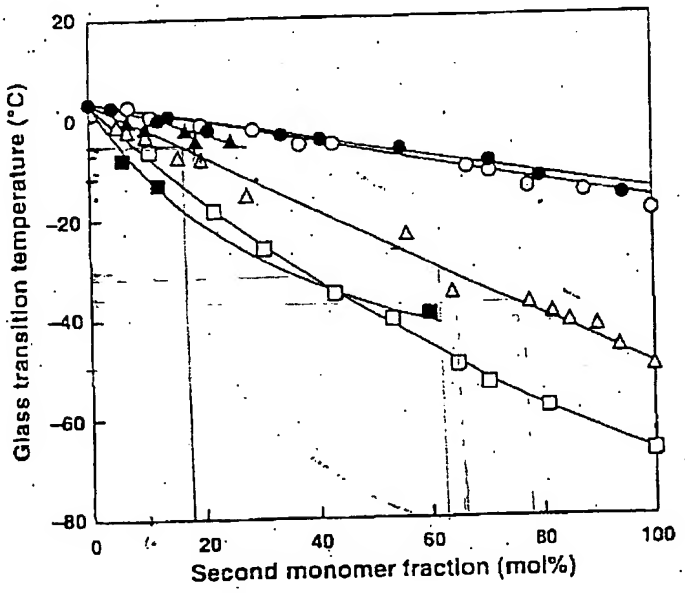


Fig. 10. Relation between glass transition temperatures (T_g) and the fraction of second monomer unit for random copolymers of (*R*)-3HB with different HA units: (●), P(3HB-*co*-3HV); (▲), P(3HB-*co*-3HHx); (■), P(3HB-*co*-3HA); (○), P(3HB-*co*-3HP); (△), P(3HB-*co*-4HB); and (□), P(3HB-*co*-6HH).

homopolymer was calculated to be as high as 1 GPa if the cross-section was corrected. Thus, P(3HB-*co*-4HB) copolymers exhibit a wide range of material properties [130,131].

Recently, it was found that *Pseudomonas* sp. 61-3 accumulated a novel random copolymer of 3HB and medium-chain-length 3-hydroxyalkanoates of carbon numbers ranging from 6 to 12 atoms [P(3HB-*co*-3HA)], from glucose and alkanoates [242]. More recently, it was found that genetically engineered *Pseudomonas* sp. 61-3 can produce a P(3HB-*co*-3HA) copolymer with high (*R*)-3HB compositions (up to 94 mol% (*R*)-3HB) from glucose [68]. In the case of P(3HB-*co*-6 mol% 3HA) films the elongation to break reached 680% with the introduction of only 6 mol% of MCL 3HA units. The resulting tensile strength was determined to be 17 MPa, indicating that the copolymerization of (*R*)-3HB units with MCL 3HA units is effective for improving the brittleness of P(3HB) films [68]. The mechanical properties of P(3HB-*co*-6 mol% 3HA) were also found to be very similar to those of low-density polyethylene, as shown in Table 3.

3.1.3. Poly[(R)-3-hydroxybutyrate]-based polymer blends

To regulate the physical properties of P(3HB), polymer blends of P(3HB) with biodegradable polymers have been investigated. Polymer blends are physical mixtures of structurally different polymers, and the mixture of two polymer forms, either homogeneous or heterogeneous phases, in amorphous regions on a microscopic scale at equilibrium. When a mixture of two polymers in the amorphous phase exists as a single phase, the blend is considered to be miscible in the thermodynamic sense. In contrast, when a mixture of two polymers separates into two distinct phases consisting primarily of the individual components, the blend is considered to be immiscible in the thermodynamic sense. The physical properties of a mixture are strongly dependent on the phase structures. Therefore, the miscibilities of P(3HB)-based polymer blends have been evaluated extensively.

Miscible blends containing P(3HB) have been formed with poly(ethylene oxide) [243–246], poly(vinyl alcohol) [247,248], atactic poly(3-hydroxybutyrate) [249–253], poly(lactide) [254,255], poly(ε-caprolactone-*co*-lactide) [256], poly(butylene succinate-*co*-butylene adipate) [257] and poly(butylene succinate-*co*-ε-caprolactone) [257]. On the other hand, mixtures of P(3HB) with poly(β-propiolactone) [258,259], poly(ethylene adipate) [258], poly(butylene adipate) [260] and poly(ε-caprolactone) [260] are all immiscible. In the case of the miscible blends of P(3HB) with atactic poly(3-hydroxybutyrate), elongation to break was increased from 5 to 500% with an increase in the content of atactic poly(3-hydroxybutyrate) content from 0 to 76 wt%, while the Young's modulus and tensile strength were both

Table 3
Comparison of PHA polymers with common plastics in properties

Sample	Melting temperature (°C)	Glass-transition temperature (°C)	Young's modulus (GPa)	Tensile strength (MPa)	Elongation to break (%)
P(3HB)	180	4	3.5	40	5
P(3HB-*co*-20 mol% 3HV)	145	−1	0.8	20	50
P(3HB-*co*-6 mol% 3HA)[a]	133	−8	0.2	17	680
Polypropylene	176	−10	1.7	38	400
Low-density polyethylene	130	−30	0.2	10	620

[a] 3HA units: 3-hydroxydecanoate (3 mol%), 3-hydroxydodecanoate (3 mol%), 3-hydroxyoctanoate (<1 mol%), 3-hydroxy-*cis*-5-dodecenoate (<1 mol%).

diminished [249]. In contrast, the addition of poly(ε-caprolactone) to P(3HB) resulted in a decrease in the Young's modulus and tensile strength without an increase in elongation to break, owing to macroscopic phase separation [260].

3.2. Biodegradability of PHA

3.2.1. Environmental degradability of PHA materials

One of the unique properties of biological PHA materials is their biodegradability in various environments. The biodegradability of PHA materials in natural environments such as soil [261], sea water [262,263] and lake water [263], has been evaluated by monitoring the properties of samples (sample dimension, molecular weight and mechanical strength), and it has been found that the rate of biodegradation of PHA materials depends on many factors, notably those related to the environment (temperature, moisture level, pH and nutrient supply) and those related to the PHA materials themselves (composition, crystallinity, additives and surface area). Electron microscopy has revealed that degradation occurs at the surface by enzymatic hydrolysis (surface erosion). The molecular weights of PHA samples remained almost unchanged during the course of biodegradation.

A number of microorganisms such as bacteria and fungi in soil, sludge and sea water excrete extracellular PHA-degrading enzymes to hydrolyze solid PHA into water-soluble oligomers and monomer, and subsequently utilize the resulting products as nutrients within cells. To evaluate the biodegradability of PHA materials and the utilizability of their degradation products, the modified MITI test (OECD guideline for testing of chemicals no.301C) has been performed in aquatic environments for PHA samples [264–267]. The time-dependent changes in the biochemical oxygen demand (BOD), weight loss (erosion) of the polyester sample and dissolved organic carbon (DOC) concentration of the test solution were measured to confirm that the PHA materials are degraded completely in aquatic environments under aerobic conditions, and that oligomers of HA units from PHA are metabolized by microorganisms in the environment.

3.2.2. Enzymatic degradability of PHA materials

3.2.2.1. Structure and properties of extracellular PHB depolymerases. Since P(3HB) is a solid polymer with a high molecular weight and is incapable of being transported through the cell wall, various microorganisms excrete extracellular PHB depolymerases that hydrolyze the solid P(3HB) into the water-soluble monomer and oligomers. Such P(3HB)-degrading microorganisms have been isolated from various environments such as soil [261,268–272], sea water [263,273,274], lake water [275], air [276], hot springs [277], compost [278] and sludge [279]. Many extracellular PHB depolymerases have been purified from different microorganisms and characterized [273,276,279–282]. The purified PHB depolymerases consist of a single polypeptide chain and their molecular weights are in the range of 37,000–60,000 [272,283–291]. Analysis of the structural genes for extracellular PHB depolymerases has shown that all enzymes are comprised of an N-terminal catalytic domain, a C-terminal substrate-binding domain, and a linker region connecting the two domains (Fig. 11). The presence of both catalytic and binding domains has been found in many depolymerizing enzymes such as cellulase [292], xylanase [292,293] and chitinase [294], which hydrolyze water-insoluble polysaccharides.

The catalytic domain contains a lipase box pentapeptide [Gly-X_1-Ser-X_2-Gly] as an active site which is common for serine hydrolase [295]. The active site serine forms a catalytic triad with an aspartate and

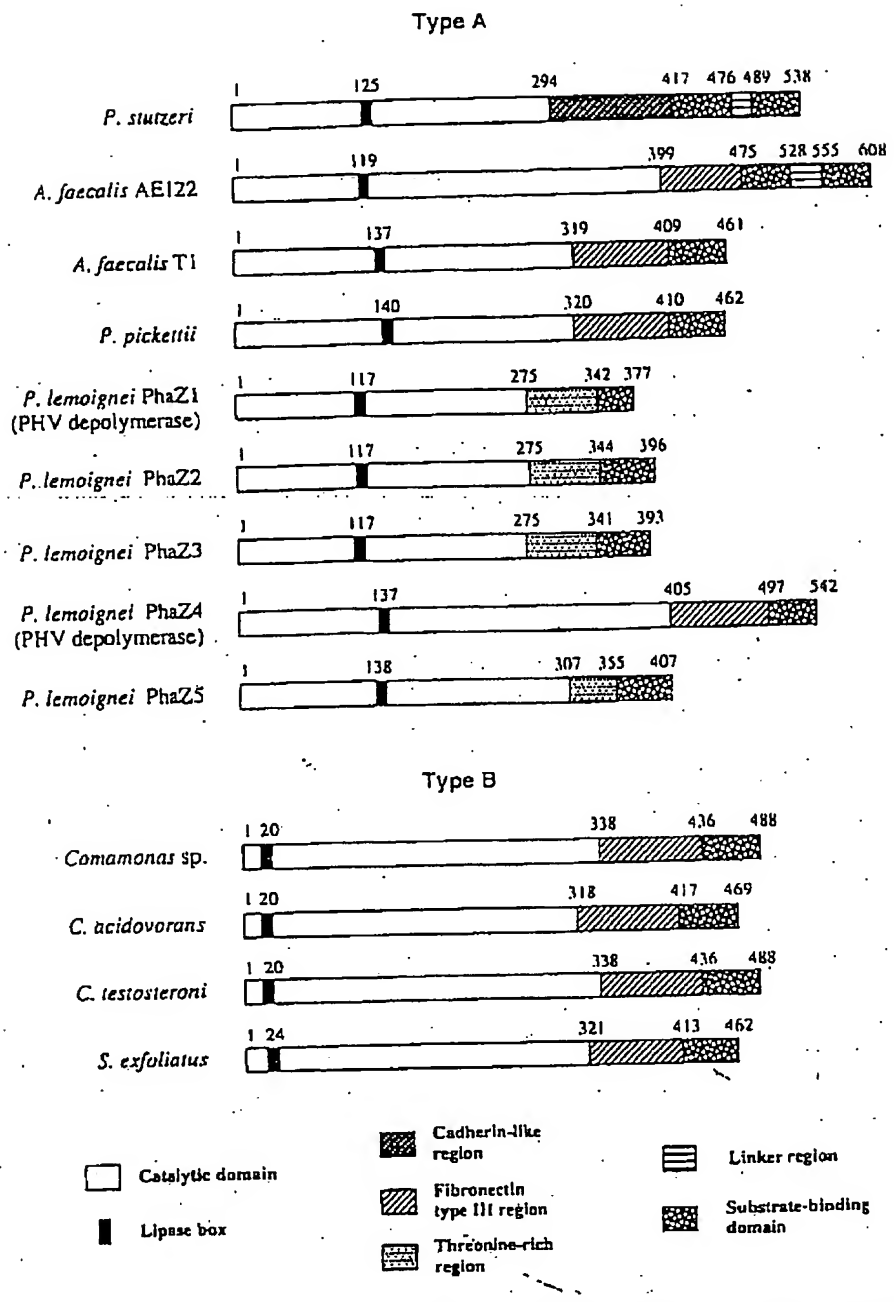


Fig. 11. Domain structure and subclasses of extracellular PHB depolymerases from various microorganisms.

a histidine, and a transient tetrahedral intermediate of the substrate carbonyl carbon is stabilized by NH groups around the histidine residue (oxyanion hole) [286,289]. As shown in Fig. 11, extracellular PHB depolymerases are classified into two types by the difference in the position of the lipase box in the catalytic domain. Type A enzymes, in which a lipase box is located in the center of the catalytic domain, are represented by *Alcaligenes faecalis* AE122, *A. faecalis* T1, *Pseudomonas lemoignei* PhaZ1, *P. lemoignei* PhaZ2, *P. lemoignei* PhaZ3, *P. lemoignei* PhaZ4, *P. lemoignei* PhaZ5 and *Pseudomonas stutzeri*, while type B enzymes, in which a lipase box is adjucent to the N-termini, are produced by *Comamonas* sp., *C. acidovorans*, *C. teststeroni* and *Streptomyces exfoliatus*.

It has been suggested that the C-terminal domain acts as a substrate-binding domain for water-insoluble P(3HB) substrate. This is because the PHB depolymerase without the C-terminal domain lost the hydrolyzing activity toward the water-insoluble P(3HB), while it retained the activity toward water-soluble (*R*)-3HB oligomers [295]. In addition, a fusion protein of putative substrate-binding domain of PHB depolymerase with glutathione S-transferase demonstrated that the substrate-binding domain moiety is essential for the adsorption of PHB depolymerase onto the surface of P(3HB) powder [296].

The linker regions connecting catalytic and substrate-binding domains have been identified in all known PHB depolymerases, and show either a fibronectin type III module, threonine-rich regions or cadherin-like domains [284,287,295,297,298]. Cell membrane proteins such as fibronectins and cadherins have been found in the linker regions of water-insoluble polymer hydrolases such as cellulases [292] and chitinases [294,299]. It has been suggested that the linker regions play a structural role in maintaining an optimal distance between the catalytic domain and substrate-binding domain.

The properties of an extracellular PHB depolymerase from *A. faecalis* have been extensively investigated. The enzymatic hydrolysis of P(3HB) by the PHB depolymerase produces as a major product 3HB dimer, and small amounts of 3HB monomer. Hydrolysis of end-labeled 3-hydroxybutyrate oligomers by the PHB depolymerase showed that the enzyme mainly cleaved the second and third ester linkages from the hydroxyl terminus [280]. However, since the enzyme also hydrolyzes cyclic oligomers, the PHB depolymerase from *A. faecalis* has *endo*-hydrolase activity in addition to *exo*-hydrolase activity [280]. A recent study on the enzymatic hydrolysis of 3HB oligomers by the PHB depolymerase demonstrated that the active site of depolymerase recognizes the sequential four monomeric units as a substrate for the hydrolysis of ester bonds in a polymer chain [300].

The rate of enzymatic erosion of P(3HB) by PHB depolymerase is strongly dependent on the concentration of the enzyme. The rate of enzymatic hydrolysis increased to a maximum value along with the concentration of PHB depolymerase, and was then followed by a gradual decrease [200,301–303]. Solid P(3HB) polymer is a water-insoluble substrate, while PHB depolymerases are soluble in water. The enzymatic degradation of P(3HB) materials by PHB depolymerase, therefore, is a heterogeneous reaction involving two steps, namely, adsorption and hydrolysis. The first step is adsorption of the enzyme onto the surface of the P(3HB) material by the binding domain of the enzyme, and the second step is hydrolysis of polymer chains by the active site of the enzyme. Accordingly, the kinetic behavior of P(3HB) hydrolysis has been accounted for in terms of a surface-enzymatic reaction that the hydrolysis of P(3HB) chains takes place by the surface reaction between adsorbed enzymes and free adsorption points on the surface [200,301–303]. In this kinetic scheme, the ratio of surface-occupation by enzyme molecules is given by the Langmuir adsorption. From the kinetic analysis on the adsorption of the enzyme onto the surface of P(3HB), it has been confirmed that the adsorption isotherms of PHB depolymerase are expressed by the Langmuir adsorption equation [304]. It has been concluded that at

low concentrations of PHB depolymerase, the majority of catalytic domains of adsorbed enzymes are able to hydrolyze P(3HB) chains on the surface, whereas at high concentrations of enzyme, the majority of catalytic domains cannot access the P(3HB) chains on the surface due to over-crowding of the substrate-binding domains on the surface of P(3HB).

3.2.2.2. Effects of chemical structures on enzymatic degradability. Biological P(3HB) is composed of only the (*R*)-3-hydroxybutyrate unit. In contrast, chemosynthetic P(3HB) is prepared by the ring-opening polymerization of β-butyrolactone (β-BL) and contains both (*R*)- and (*S*)-3HB units. The stereocomposition and tacticity of chemosynthetic P(3HB) can be regulated by varying both the feed ratio of (*R*)- and (*S*)-β-BL and the type of catalyst [305–313]. Several researchers have investigated the stereoselectivity of PHB depolymerase for the hydrolysis of poly(3-hydroxybutyrate) [305–313].

The chemosynthetic P(3HB) samples containing both monomeric units of (*R*)- and (*S*)-3HB have been shown to be hydrolyzed by PHB depolymerases, while P(3HB) samples with high (*S*)-3HB fractions (over 92 mol%) were hydrolyzed to only a small extent by the PHB depolymerases [305,306]. The enzymatic hydrolysis of biological P(3HB) by PHB depolymerases produces water-soluble products composed of monomer and/or dimer of (*R*)-3-hydroxybutyrate. In contrast, after enzymatic degradation, chemosynthetic P(3HB) samples gave 3HB oligomers as degradation products in addition to 3HB monomer and dimers, suggesting that the PHB depolymerase poorly hydrolyzes the ester bonds connecting the (*S*)-3HB units [305,309]. The rate of enzymatic erosion of chemosynthetic P(3HB) samples containing both monomeric units of (*R*)- and (*S*)-3HB is strongly dependent on both the stereocomposition and on the tacticity of the sample. Recently, Bachmann and Seebach [300] investigated the stereoselectivity of PHB depolymerase from *A. faecalis* using the stereoisomers of 3HB oligomer, and demonstrated that the PHB depolymerase hydrolyzes only the ester bond between sequential (*R*)-3HB units.

The substrate specificity of PHB depolymerase has been investigated using various PHA homopolymers [200,314]. Fig. 12 shows the enzymatic degradabilities of 12 types of aliphatic polyester by three types of extracellular PHB depolymerase. Although the rates of hydrolysis were different among the three PHB depolymerases, all of the PHB depolymerases hydrolyzed the same kinds of film, P(3HB), poly(3-hydroxypropionate), poly(4-hydroxybutyrate), poly(ethylene succinate) and poly(ethylene adipate) [314]. The five enzymatically degradable polyesters have some common chemical structures: the number of carbon and oxygen atoms between the two carbonyl groups in the backbone is 3 or 4, while the side chain is methyl carbon or hydrogen. Thus, PHB depolymerases show relatively narrow substrate specificities for PHA hydrolysis. From the adsorption study using PHB depolymerases [304] and the fusion proteins of substrate-binding domains of PHB depolymerases with glutathione S-transferase [289,291,296,298,314], it is seen that PHB depolymerases are capable of binding on the surfaces of both enzymatically degradable and non-degradable polyesters, while they hardly bind to the polysaccharides of Avicel or chitin [314]. These results suggest that there are some specific interactions based on molecular recognition between the substrate-binding domain and the surface of polyesters, while the binding specificity of the substrate-binding domain is broad compared with the substrate specificity of the active site in the catalytic domain.

The effects of chemical structures of the second monomer units and copolymer compositions on the rate of enzymatic erosion have been examined through the enzymatic degradation of the solution-cast films of random copolymers of (*R*)-3HB with various hydroxyalkanoates units in the presence of PHB depolymerase [131,236,237,315–318]. The enzymatic degradation of solution-cast films of these PHA

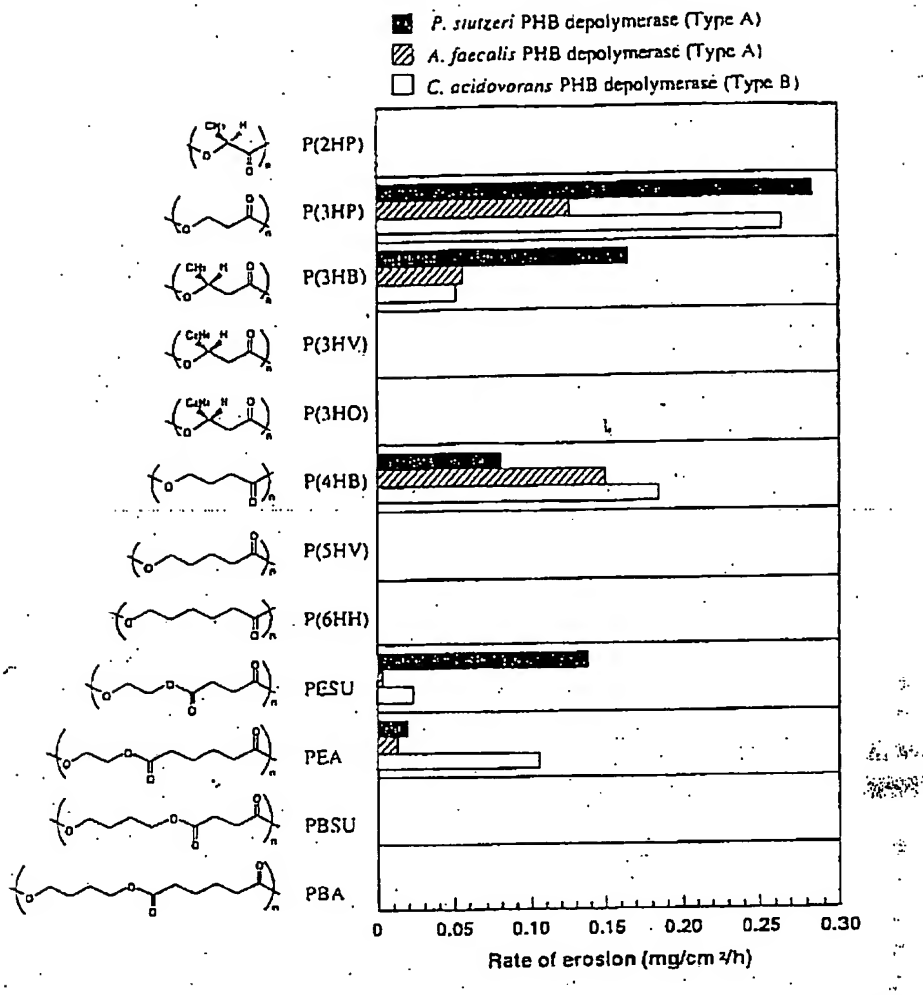


Fig. 12. Rates of enzymatic erosion for 12 polyester films at 37°C in aqueous solution (pH 7.4) containing PHB depolymerases from *A. faecalis* ($PhaZ_{Afa}$), *Pseudomonas stutzeri* ($PhaZ_{Pst}$) or *Comamonas acidovorans* ($PhaZ_{Cac}$).

copolymers was performed in aqueous solution of purified PHB depolymerase from *A. faecalis* at 37°C. The rate of enzymatic erosion on the solution-cast PHA films increased markedly with an increase in the fraction of second monomer units up to 10–20 mol% reaching maximum value followed by a decrease in the erosion rate. The highest rates for enzymatic erosion were 5–10 times larger than the rate for the P(3HB) homopolymer film.

The structures and compositions of water-soluble products of PHA copolymers during enzymatic degradation by PHB depolymerase from *A. faecalis* have been characterized using high-performance liquid chromatography and NMR analysis [131,236,237,315–318]. The water-soluble products of random copolymers by the PHB depolymerase showed a mixture of monomers and several oligomers of (*R*)-3HB and hydroxyalkanoates units. The water-soluble degradation products of random copolymers have some common chemical structures: the carboxyl-termini of the water-soluble products were

always the monomeric units with main-chain lengths of 3 or 4 carbon atoms, while the hydroxyl-termini were always the monomeric units having a side-chain length ranging from 0 to 2 carbon atoms. A schematic model for enzymatic hydrolysis of ester linkages in a polymer (PHA) chain by PHB depolymerase from *A. faecalis* has been proposed by Abe et al. [316], and is shown in Fig. 13.

The active site of the catalytic domain in PHB depolymerase recognizes at least three monomeric units as the substrate. The rate of enzymatic hydrolysis of an ester bond may be strongly dependent on the chemical structure of the second and third monomeric units in the polyester chain. The catalytic site may bind the substrate by interacting with the carbonyl group in the hydroxyl-terminated R_1 unit in order to assist the hydrolysis reaction of the second ester bond with the active site of the catalytic domain. The length of the main carbon chain of the second monomer R_2 unit may be limited to 3 or 4 carbon atoms because of a facile binding of substrate by the active site. On the other hand, the chemical structure of the third R_3 monomer unit may affect the accessibility of the ester bond to the active site of PHB depolymerase. When the third monomeric unit has a sterically bulky side-chain such as (*R*)-3HV and (*R*)-3HHx units, PHB depolymerase may hardly attack the ester bond. Consequently, the hydrolysis rate for ester bonds by PHB depolymerase decreases as the side-chain length of a hydroxyalkanoate unit increases.

3.2.2.3. Effects of solid-state structures on enzymatic degradability. P(3HB) is a semicrystalline thermoplastic with a melting temperature around 180°C, and can be processed by conventional extrusion and molding equipment. The melt-crystallized films of P(3HB) exhibit large banded spherulites of diameter 50–500 μm, and the spherulitic morphologies and degree of crystallinities are dependent upon the crystallization conditions. The rate of enzymatic hydrolysis for melt-crystallized P(3HB) films by PHB depolymerase from *A. faecalis* decreased with an increase in the crystallinity of the P(3HB)

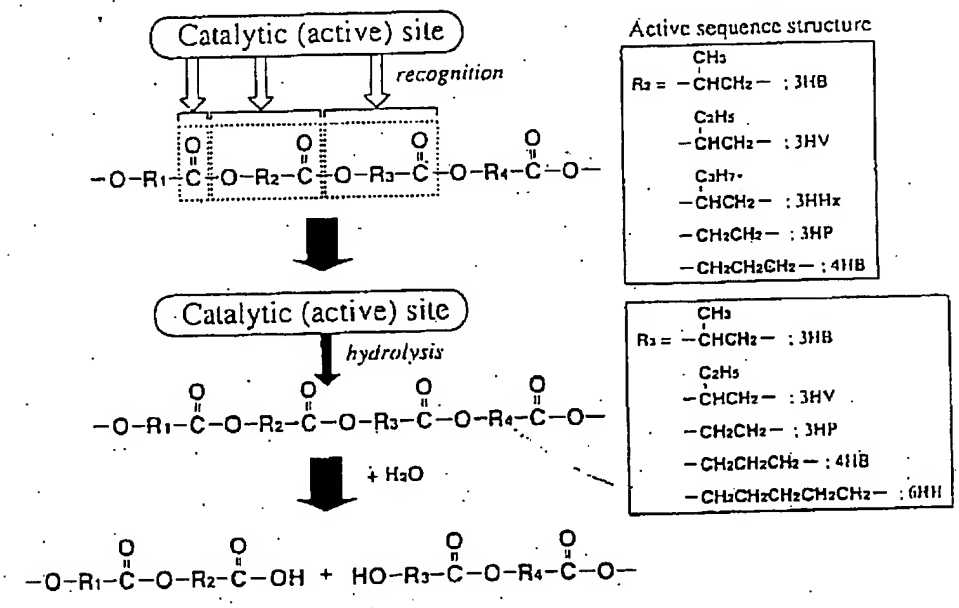


Fig. 13. A schematic model for the enzymatic hydrolysis of an ester bond in various sequences by extracellular PHB depolymerase.

film, while the size of spherulites did not significantly affect the rate of hydrolysis [319]. In addition, it was demonstrated that the rate of enzymatic degradation for P(3HB) chains in an amorphous state was approximately 20 times higher than the rate for P(3HB) chains in a crystalline state [319]. This shows that the rate of biodegradability of P(3HB) materials can be regulated by varying the degree of crystallinity.

The size of the crystal for melt-crystallized P(3HB) films also varied with crystallization conditions. Tomasi et al. [320] prepared melt-crystallized P(3HB) films with different crystal sizes and examined the rates of enzymatic hydrolysis by PHB depolymerase isolated from *P. lemoignei*. They reported that the rate of enzymatic erosion of melt-crystallized P(3HB) films with the PHB depolymerase decreased with an increase in the average size of the P(3HB) crystal.

Koyama and Doi [321] prepared melt-crystallized films of various random copolymers of (*R*)-3HB with different HA units and studied the rates of enzymatic erosion on the surface of films by PHB depolymerase from *A. faecalis* (Fig. 14). The erosion rates for P(3HB-*co*-3HV) films were several times higher than the rates of P(3HB) homopolymer films with the same degree of crystallinity. Therefore, the significant difference in the erosion rates for melt-crystallized films of P(3HB) homopolymer and P(3HB-*co*-3HV) copolymers could not be explained only in terms of the degree of crystallinity and the average size of the crystal structures.

The thickness of crystalline lamellae for melt-crystallized films also varied with crystallization conditions. The rates of enzymatic degradation for melt-crystallized films of PHA copolymers with both different crystallinities and different lamellar thicknesses have been investigated by Abe et al. [240]. The rates of enzymatic erosion for melt-crystallized copolyester films significantly decreased as the degree of crystallinity was increased (Fig. 14). It was suggested that the PHB depolymerase predominantly hydrolyzes polymer chains in the amorphous phase and then, subsequently erodes the crystalline phase. In addition, the enzymatic erosion rate for the crystalline phase in polyester films was determined from overall erosion rates and crystallinities. The enzymatic erosion rates of crystalline phases in polyester films decreased with an increase in the lamellar thickness (see Fig. 15).

To elucidate the mechanism for enzymatic degradation of the crystalline region for P(3HB) by PHB depolymerase, the enzymatic degradation of single crystals of P(3HB) with PHB depolymerases isolated from bacteria and fungi have been studied [216,217,322–324]. The results are summarized in Fig. 16. Since the lamellar thicknesses of single crystals and the molecular weights of P(3HB) chains remain unchanged during enzymatic degradation, the single crystals were enzymatically hydrolyzed, preferentially at the crystal edges (*ac* plane) and ends (*bc* plane), rather than the chain-folding surfaces (*ab* plane) of the single crystals. Many narrow cracks and small crystal fragments along the crystal long axis corresponding to the crystallographic *a* axis were produced from P(3HB) single crystals by the enzymatic reaction, independent of both surface morphologies of single crystals and the types of PHB depolymerase (Fig. 16c). The PHB depolymerase molecules bind on the entire surface of P(3HB) single crystals (Fig. 16b). The binding of PHB depolymerase on the surface of P(3HB) lamellar crystal may cause an increase in the mobility of polyester chains along the crystal edge, resulting in the formation of disordered P(3HB) chains resembling the polymer chains in the amorphous phase which are facilely attacked by the active site of the enzyme. In the cases of single crystals for P(3HB) copolyesters; P(3HB-*co*-3HV), P(3HB-*co*-3HHx), P(3HB-*co*-4HB) and P(3HB-*co*-6HH), the enzymatic degradation mechanism is the same as in the case of the P(3HB) single crystal [218,235].

From scanning electron microscopic observations, the surfaces of melt-crystallized copolyester films after enzymatic degradation were apparently blemished by the action of PHB depolymerase, and a

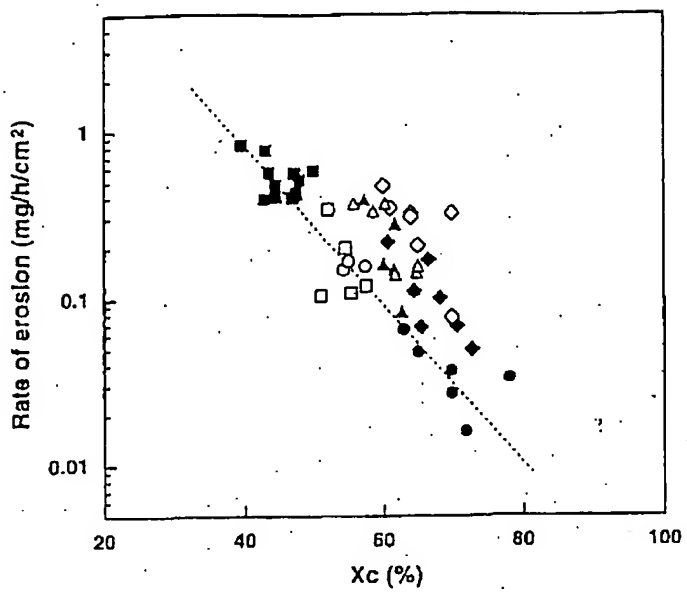


Fig. 14. Relation between the rate of enzymatic hydrolysis and the degree of crystallinity (X_c) for the melt-crystallized films of random copolymers of (*R*)-3HB with different HA units: (●), P(3HB); (◆), P(3HB-*co*-6 mol% 3HV); (◇), P(3HB-*co*-16 mol% 3HV); (○), P(3HB-*co*-8 mol% 3HHx); (▲), P(3HB-*co*-8 mol% 4HB); (△), P(3HB-*co*-10 mol% 4HB); (■), P(3HB-*co*-10 mol% 6HH); and (□), P(3HB-*co*-5 mol% LA).

ringed texture for the spherulites was detected. Two different types of plane were observed on the surface of the PHA spherulites after enzymatic degradation, i.e. a smooth plane and a rough plane. These planes existed alternately along the radial direction of the spherulite [240]. This shows that the lamellar stacks on the surface of melt-crystallized films are also hydrolyzed preferentially at the crystal edges by PHB depolymerase.

As mentioned earlier, the binding specificity of the substrate-binding domain is broad compared with the substrate specificity of the catalytic domain. As the first step of enzymatic hydrolysis of a solid polymer, the binding of PHB depolymerase to the substrate is essential for the hydrolysis reaction to proceed. The solid-state structures of the polymers also affect the adsorption reaction of the PHB depolymerase. Zinc-based catalysts produce an amorphous atactic P[(*R*,*S*)-3HB] from racemic β-BL. Little erosion occurred on the surface of the amorphous atactic P[(*R*,*S*)-3HB] sample with PHB depolymerase. However, when amorphous P[(*R*,*S*)-3HB] was blended with crystalline biological P[(*R*)-3HB], the enzymatic hydrolysis of atactic P[(*R*,*S*)-3HB] chains took place [253]. Water-soluble products liberated from P[(*R*)-3HB]/atactic P[(*R*,*S*)-3HB] blend films were a mixture of monomer and oligomers of (*R*)- and (*S*)-3HB units, indicating that the atactic P[(*R*,*S*)-3HB] component is hydrolyzed by PHB depolymerase in the presence of the stereoregular P[(*R*)-3HB] component. It has been found that the presence of crystalline components such as P(3HB-*co*-3HV), poly(ϵ-caprolactone), poly(lactide) and poly(pivalolactone) by blending [325,326] or block-copolymerizing [327] with atactic P[(*R*,*S*)-3HB] induce the enzymatic hydrolysis of the atactic P[(*R*,*S*)-3HB] molecule by PHB depolymerase. These results suggest that the PHB depolymerase is liable to bind to the surface of stable crystalline lamellae.

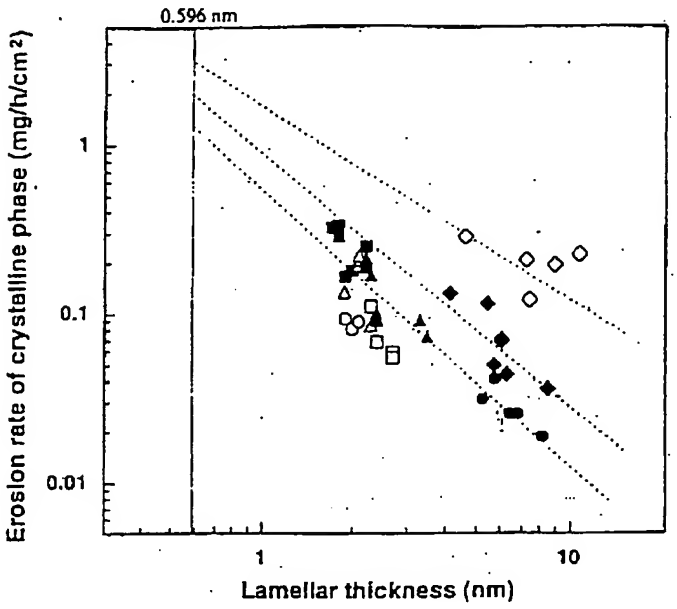


Fig. 15. Relationship between the erosion rate of crystalline phase (R_c) and the lamellar thickness (l_c): (●), P(3HB); (◆), P(3HB-*co*-6 mol% 3HV); (◇), P(3HB-*co*-16 mol% 3HV); (○), P(3HB-*co*-8 mol% 3HHx); (▲), P(3HB-*co*-8 mol% 4HB); (△), P(3HB-*co*-10 mol% 4HB); (■), P(3HB-*co*-10 mol% 6HH); and (□), P(3HB-*co*-5 mol%LA).

but it hardly binds to the surface of mobile polymer chains in an amorphous state above the glass-transition temperature. The binding domain of PHB depolymerase adsorbs selectively to the crystalline phase on the film surface, and then the catalytic domain hydrolyzes predominantly the P(3HB) chains located in the amorphous regions on the surface.

The adsorption of PHB depolymerase to single crystals has been investigated using immuno-gold labeling techniques (see Fig. 16b). On both the surfaces of P(3HB) homopolymer [216,217] and copolymer [218,235] single crystals, the binding of PHB depolymerase showed a homogeneous distribution on the surfaces of single crystals, which suggests that the substrate-binding domain contributes to the adsorption of enzyme on P(3HB) crystals without site specificity. However, the concentrations of the adsorbed enzyme on the surfaces of copolymer single crystals were found to be lower than those on the P(3HB) homopolymer single crystal. PHB depolymerase molecules may not bind tightly to the irregular surfaces of copolymer crystals since the copolymer chains with second monomer units have loose loop foldings on the surface of the crystals.

To this end, it is clear that the degradation of PHA is affected by many factors such as monomer composition, molecular weight and degree of crystallinity. An increased understanding of PHA microstructure will contribute further to the elucidation of its degradation mechanism.

4. Conclusion

Bacterial PHA first received commercial attention way back in the early 1960s from an American

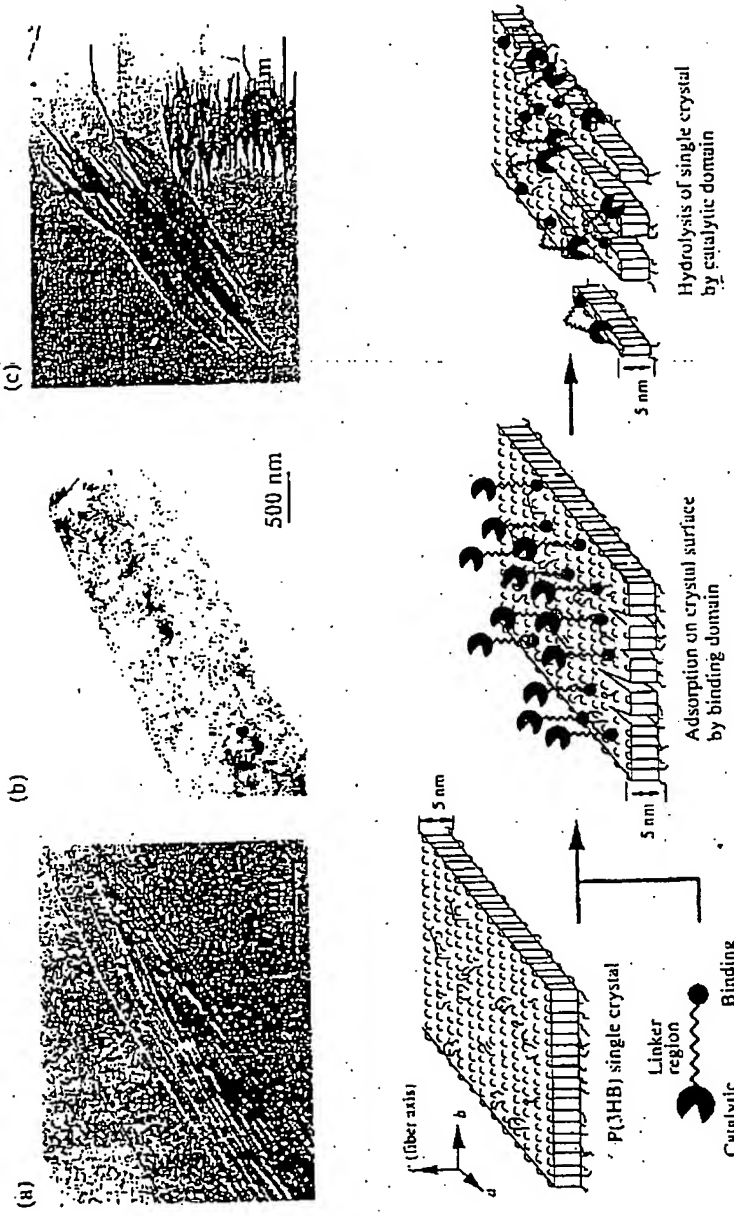


Fig. 16. A schematic model of enzymatic hydrolysis of P(3HB) single crystal by PHB depolymerase consisting of binding and catalytic domains: (a) structure and morphology of P(3HB) single crystal; (b) binding of PHB depolymerase molecules onto the entire surface of P(3HB) single crystal; and (c) enzymatic hydrolysis of the P(3HB) crystal edge.

company, W.R. Grace, but apparently was discontinued due to some technical problems [328]. The major petroleum crisis of the 1970s then motivated a British company, ICI Bioproducts, (now known as Zeneca Bio Products) to get involved with this bacterial polymer. It was anticipated that the potential development of plastic materials from renewable resources would reduce the dependency on petroleum prices [329]. On the contrary, petroleum prices did not increase as anticipated and petrochemical-based plastics remain the cheapest available plastics. However, environment-friendly features such as biodegradabilities and biocompatibilities, which are lacking in conventional plastics, have continued as the commercial interest in PHA [101]. In the early 1990s, despite higher costs of production, a German company Wella started using PHA as a packaging material for some of its hair-care products.

In response to an increased awareness of global environmental problems, PHA is gaining serious attention as a potential substitute for non-biodegradable polymers. Envisaging the potential for a new generation of environmentally friendly plastics, Metabolix Inc, of Cambridge, Massachussets, has now taken up the challenge to further develop efficient technologies for PHA production (http://metabolix.com./). Traditionally, synthetic polymers were designed for durability and resistance to the environment and, therefore, tend to accumulate in landfills. With growing concerns for the environment, synthetic polymers containing hydrolyzable and/or oxidizable groups along the main chain are also being developed. It can be anticipated that in the future, PHA will have to compete with this class of synthetic biodegradable polymers. In the medical field, polymers such as poly([*S*]-lactic acid) and poly(glycolic acid) (PGA) are also being developed as potential bioabsorbable materials. Despite competition from these synthetic polymers, the diverse nature of PHAs will ensure its preference for particular situations. For example, in tissue engineering of heart valves, it is not possible to create a functional trileaflet heart valve scaffold from PGA. On the other hand, P(3HB-*co*-3HHx) and P(4HB)-based tissues are characterized by supraphysiologic mechanical strength, which is an important finding for surgical applications [330]. In addition, detailed structural analysis of various PHAs has provided us with further insights about their physical properties and degradation mechanisms. As a result, it is now possible to design PHAs with physical properties that can biodegrade in a predetermined time and manner.

Based on the wealth of information that has been gathered to date, PHA seems to hold great promise as an environmentally friendly polymeric material. In a world with shrinking petroleum reserves and increasing environmental issues, PHA is definitely a potential candidate that deserves further exploration. The biocompatible nature of PHA and its potential applications in the medical field should also not be overlooked.

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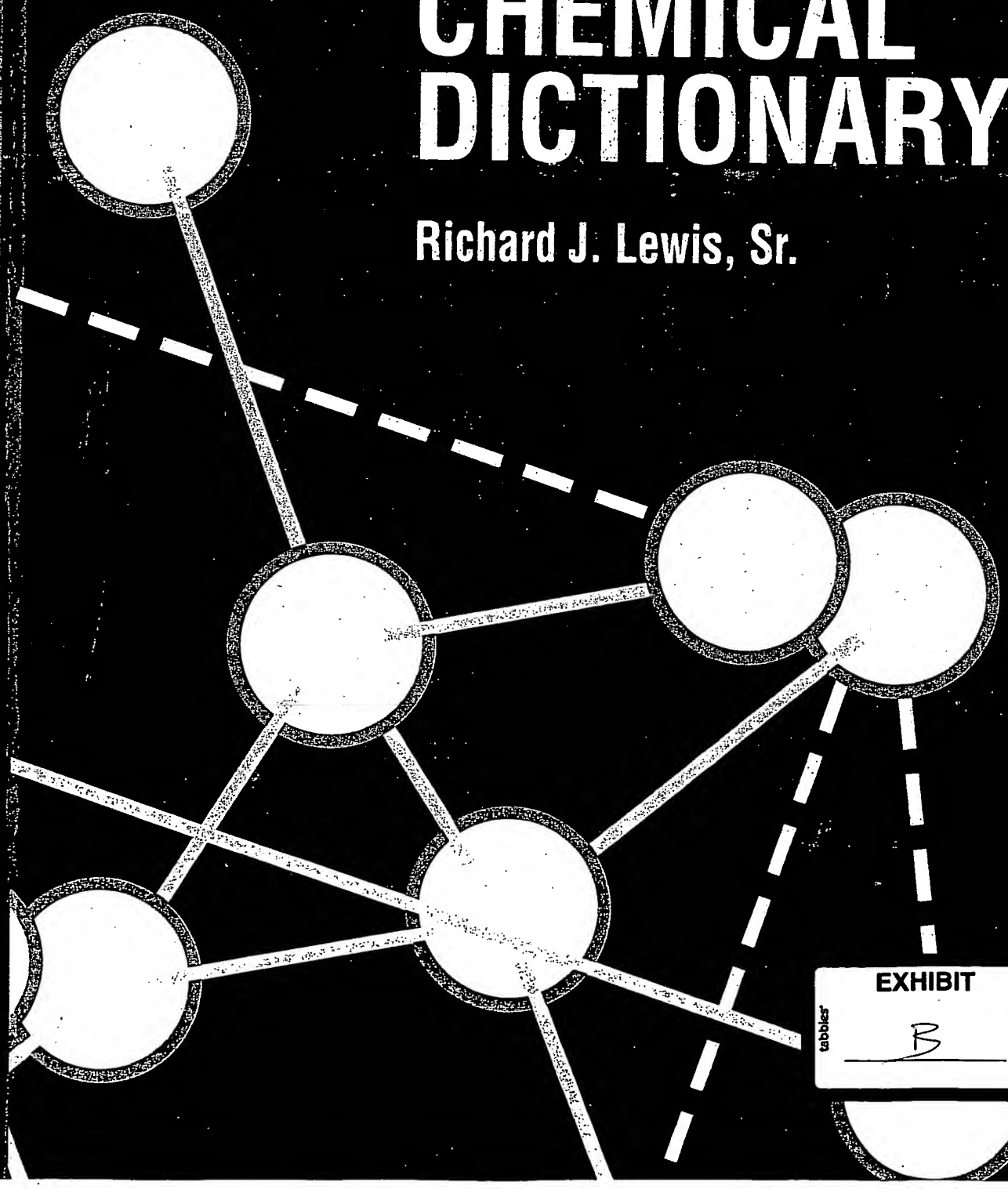

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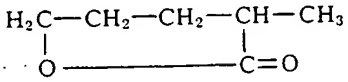
Derivation: Distilled from roots and rhizome of Valeriana officinalis.
Use: Tobacco perfume, industrial odorant, flavors.

valeric acid. (valerianic acid; n-pentanoic acid). CAS: 109-52-4. $CH_3(CH_2)_3COOH$.
Properties: Colorless liquid, penetrating odor and taste, d 0.9394 (20/4C), bp 185.4C, refr index 1.4081 (20C), vap press 0.08 mm Hg (20C), fp −34C, flash p (OC) 205F (96C), soluble in water, soluble in alcohol and ether, undergoes reactions typical of normal monobasic organic acids. Combustible.
Derivation: With other C_5 acids by distillation from valerian, by oxidation of n-amyl alcohol, numerous essential oils.
Grade: Technical, reagent.
Hazard: Toxic by ingestion, strong irritant to skin and tissue.
Use: Intermediate for flavors and perfumes, ester-type lubricants, plasticizers, pharmaceuticals, vinyl stabilizers.
See also isovaleric acid.

valeric aldehyde. See n-valeraldehyde.

γ-valerolactone. CAS: 108-29-2.



Properties: Colorless liquid, d 1.0518 (25/25C), bp 205–206.5C, crystallizing point −37C, flash p (COC) 205F (96C), refr index 1.4301 (25C), surface tension 30 dynes/cm (25C), viscosity 2.18 cP (25C), pH (anhydrous) 7.0 (pH of 10% solution in distilled water 4.2). Miscible with water and most organic solvents, resins, waxes, etc.; slightly miscible with zein, beeswax, petrolatum; not miscible with anhydrous glycerin, glue, casein, arabic gum, and soybean protein. Combustible.
Use: In dye baths (coupling agent), brake fluids, cutting oils, and as solvent for adhesives, insecticides, and lacquers.

valine. (α-aminoisovaleric acid). CAS: (L-) 72-18-4. $(CH_3)_2CHCH(NH_2)COOH$. An essential amino acid.
Properties: White, crystalline solid; soluble in water; very slightly soluble in alcohol; insoluble in ether; shows the following optical isomers:
DL-valine: mp 298C with decomposition.
D-valine (natural isomer): mp 315C with decomposition.
L-valine: mp 293C with decomposition.
Derivation: Hydrolysis of proteins, synthesized by the reaction of ammonia with α-chloroisovaleric acid. Available commercially as D-, L-, or DL-valine.
Use: Dietary supplement, culture media, biochemical and nutritional investigations.

"Valium" [*Hoffmann-LaRoche*]. TM for diazepam.

valone. (2-isovaleryl-1,3-indanedione). $C_{14}H_{14}O_3$.
Properties: Yellow, crystalline solid; mp 68C; insoluble in water; soluble in common organic solvents; blood anticoagulant.
Hazard: Toxic by ingestion.
Use: Pesticide, rodenticide.

vanadic acid. (1) meta-HVO_3, (2) ortho-H_3VO_4, (3) pyro-$H_4V_2O_7$. These acids apparently do not exist in the pure state, but are represented in the various alkali and other metal vanadates. Ordinarily, vanadic acid implies vanadium pentoxide (vanadic acid anhydride).

vanadic acid anhydride. See vanadium pentoxide.

vanadic sulfate. See vanadyl sulfate.

vanadic sulfide. See vanadium sulfide.

vanadinite. $Pb_5Cl(VO_3)$. A natural chlorovanadate of lead.
Properties: Ruby red, orange red, brown, yellow; luster resinous to adamantine; Mohs hardness 3; d 6.7–7.1; soluble in strong nitric acid.
Occurrence: New Mexico, Arizona, Africa, Scotland, the former USSR.
Use: Ore of vanadium and lead.

vanadium. CAS: 7440-62-2. V. Metallic element having atomic number 23, group VB of the periodic system, aw 50.9414, valences = 2, 3, 4, 5,; two natural isotopes.
Properties: Silvery-white ductile solid; insoluble in water; resistant to corrosion, but soluble in nitric, hydrofluoric, and concentrated sulfuric acids; attacked by alkali, forming water-soluble vanadates. D 6.11, mp 1900C, bp 3000C, acts as either a metal or a nonmetal and forms a variety of complex compounds.
Source: Not found native: principal ores are patronite, roscoelite, carnotite, and vanadinite. Also from phosphate rock (Idaho, Montana, Arkansas).
Occurrence: Colorado, Utah, New Mexico, Arizona, Mexico, and Peru.
Derivation: (1) Calcium reduction of vanadium pentoxide yields 99.8+% pure ductile vanadium; (2) aluminum, cerium, etc., reduction produces a less pure product; (3) solvent extraction of petroleum ash or ferrophosphorus slag